SHIP (SH2-containing inositol 5′-phosphatase) is a hematopoietic restricted enzyme responsible for the hydrolysis of the phosphatidylinositol 3-kinase-generated second messenger PI-3,4,5-P3 to PI-3,4-P2 and, thereby, negatively regulates cell survival, proliferation and differentiation. Herein, we demonstrate a role for SHIP in the differentiation and function of dendritic cells (DCs). We found that SHIP restrains in vitro generation and survival of bone marrow derived DCs cultured with granulocyte macrophage colony stimulating factor (GM-CSF) or fms-like tyrosine kinase 3 ligand (Flt3L). These results are consistent with the in vivo finding that SHIP-deficient mice have increased numbers of splenic DCs. We provide evidence that Ship−/- GM-CSF-derived DCs (GM-DCs) have impaired ability to activate T cells – a defect associated with deficient DC maturation and interleukin-12 (IL-12) production in response to Toll-like receptor (TLR) agonists. Reduced antigen (Ag)-specific T cell activation was associated with defective Th1 cell induction in vitro and in vivo. SHIP’s role is more restricted in Flt3L-derived DCs (FL-DCs) since the functional abnormalities of Ship−/- FL-DCs, leading to reduced DC maturation and Ag-specific T cell proliferation, are limited to MyD88-independent TLR activation pathways. Thus, we conclude that the function of SHIP in DC biology depends on the derivation context and the nature of the activating pathogen.

Next, we evaluated the role of Ship−/- DCs in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis previously thought to primarily depend on a Th1-mediated immune response. We found that SHIP-deficient mice develop EAE despite reduced DC-induced T cell activation. Notwithstanding evidence of a disease suppressive environment, we show evidence that Ship−/- mice have robust clinical symptoms after EAE induction resulting from enhanced production of Ag-specific IgMs in vivo.

In addition to T cell activation, under certain conditions DCs may suppress T cell proliferation using a variety of mechanisms. We show Ship−/- GM-DCs, despite
expressing high levels of the T cell suppressing enzyme arginase 1, do not have enhanced suppressive ability. Intriguingly, in contrast to WT GM-DCs, we found that Ship-/- GM-DCs do not use interferon gamma-induced nitric oxide production to suppress T cell proliferation but rather an alternative contact-dependent suppressive mechanism.
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<tbody>
<tr>
<td>-/-</td>
<td>functionally null alleles</td>
</tr>
<tr>
<td>+/-</td>
<td>functionally wild type alleles</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>Ag</td>
<td>antigen</td>
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<tr>
<td>Akt</td>
<td>RAC-alpha serine/threonine-protein kinase (aka PKB)</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
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<td>Arg1</td>
<td>arginase 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BDCA</td>
<td>blood DC antigen</td>
</tr>
<tr>
<td>BEC</td>
<td>[S]-[2-boronoethyl]-L-cysteine-HCl</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>c</td>
<td>cell or cell equivalents</td>
</tr>
<tr>
<td>CBA</td>
<td>cytometric bead array</td>
</tr>
<tr>
<td>CCL</td>
<td>chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional dendritic cell</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>c-fms</td>
<td>M-CSF receptor</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CFU-S</td>
<td>colony forming unit spleen</td>
</tr>
<tr>
<td>c-kit</td>
<td>cellular kit (stem cell/steel factor receptor tyrosine kinase)</td>
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</table>
CLP common lymphoid progenitor
CLR C-type lectin receptor
CMP common myeloid progenitor
CNS central nervous system
CpG DNA CpG-dinucleotides
cpm counts per minute
Cre cyclization recombination
CTL cytotoxic T lymphocyte
DC dendritic cell
DCAL-1 DC-associated lectin-1
DCIR DC immunoreceptor
DC-SIGN DC-specific ICAM 3-grabbing non-integrin
DECTIN-1 DC-associated molecule-1
DN double negative
dsRNA double stranded ribonucleic acid
E embryonic day
EAE experimental autoimmune encephalomyelitis
ECL enhanced chemiluminescence
eIF2α eukaryotic initiation factor 2 alpha
ELISA enzyme-linked immunosorbent assay
Erk extracellular signal-related kinase
ETP early T cell progenitors
FACS fluorescence activated cell sorting
FCS fetal calf serum
FITC fluorescein isothiocyanate
FL-DC Flt3L-derived DC
Flt3 fms-like tyrosine kinase 3
Flt3L Flt3 ligand
Foxp3 forkhead box P3
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GM-CSF granulocyte macrophage colony stimulating factor
GM-DC: GM-CSF-derived DC
GMP: granulocyte-macrophage progenitor
GPI: glycophasphatidylinositol
GvHD: graft-versus-host disease
HBSS: Hank’s balanced salt solution
HF: Hank’s balanced salt solution containing 5% FCS
HPV: human papillomavirus
HRP: horse radish peroxidase
HSC: hematopoietic stem cell
IkB: inhibitor of κB
ID2: inhibitor of DNA binding 2
IDO: indoleamine 2,3-dioxygenase
IFN: interferon
Ig: immunoglobulin
IKK: inhibitor of NFκB kinase
IL: interleukin
IMC: inflammatory monocyte
IMDM: Iscove’s modified Dulbecco’s medium
iNOS: inducible nitric oxide synthase
Inpp5d: inositol polyphosphate-5-phosphatase d (SHIP gene name)
IP: intraperitoneal
IP4: inositol-1,3,4,5-tetrakisphosphate
IPTG: isopropyl β-D-1-thiogalactopyranoside
IRAK: IL-1 receptor-associated kinase
IRF: interferon regulatory factor
ITAM: immunoreceptor tyrosine based activation motif
Itgax: integrin alpha X (aka CD11c)
ITIM: immunoreceptor tyrosine based inhibitory motif
IV: intravenous
JNK: JUN N-terminal kinase
kDa: kiloDalton
KO knock out
LAP latency associated peptide
LBP lipopolysaccharide binding protein
LC Langerhans cell
LFA-1 integrin lymphocyte function-associated antigen 1
Lin hematopoietic lineage maturation markers
LN lymph node
L-NMMA NG-monomethyl-L-arginine
loxP locus of X-over P1 (Cre target sequence)
LP lamina propria
LPS lipopolysaccharide
LRR leucine rich repeat
LSK lin-Sca-1+ckit+
LysMCre Lyzs promoter-driven Cre-expressing transgene
MΦ macrophages
M1 classically activated macrophage
M2 alternatively activated macrophage
mAb monoclonal antibody
MAL MyD88-adapter-like (also known as TIRAP)
MALP-2 mycoplasmal MΦ-activating lipopeptide-2kD
MAPK mitogen activated protein kinase
MBP myelin basic protein
M-CSF macrophage colony stimulating factor
MCSFR macrophage colony stimulating factor receptor
Mda5 melanoma differentiation associated gene 5
mDC myeloid dendritic cell
MDSC myeloid derived suppressor cells
MEK1/2 MAPK/ERK kinase 1/2
MEP megakaryocyte-erythrocyte progenitor
MFI mean fluorescence intensity
MHC major histocompatibility complex
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MHCI</td>
<td>major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHCII</td>
<td>major histocompatibility complex class II</td>
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<tr>
<td>MIP3α</td>
<td>macrophage inflammatory protein 3 alpha (aka CCL20)</td>
</tr>
<tr>
<td>MIP3β</td>
<td>macrophage inflammatory protein 3 beta (aka CCL19)</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed leukocyte reaction</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MPP</td>
<td>multipotent progenitors</td>
</tr>
<tr>
<td>MR</td>
<td>mannose receptor</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>MTG</td>
<td>monothioglycerate</td>
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<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
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<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>natural killer T cell</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod like receptor</td>
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<td>NOS2</td>
<td>nitric oxide synthase 2 (aka iNOS)</td>
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<td>OTII</td>
<td>allele for ovalbumin-specific T cell receptor expressed on CD4+ T cells</td>
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<tr>
<td>pAb</td>
<td>polyclonal antibody</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>Pam3Cys</td>
<td>tripalmitoyl-S-glycercylecysteine</td>
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<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
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<td>PDK1</td>
<td>PI-dependent protein kinase 1</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>PI3K</td>
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<td>phosphatidylinositol-3,4,5-trisphosphate</td>
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<td>protein kinase R</td>
</tr>
<tr>
<td>PLCγ</td>
<td>phosphatidyl lipase c gamma</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
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<tr>
<td>poly I:C</td>
<td>polyinosine:cytosine</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patch</td>
</tr>
<tr>
<td>pre-DC</td>
<td>precursor dendritic cell</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptors</td>
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<td>PTB</td>
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<td>receptor-interacting protein</td>
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<tr>
<td>rm</td>
<td>recombinant mouse</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RT-PCR</td>
<td>reverse transcription - polymerase chain reaction</td>
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<td>S</td>
<td>serine</td>
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<td>Full Form</td>
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</tr>
<tr>
<td>SAPK</td>
<td>stress activated protein kinase</td>
</tr>
<tr>
<td>SARM</td>
<td>sterile α-and armadillo-motif-containing protein</td>
</tr>
<tr>
<td>SC</td>
<td>sub-cutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SF</td>
<td>steel factor (aka stem cell factor)</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
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<td>SH2</td>
<td>Src homology 2</td>
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<td>Src homology 3</td>
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<td>colloquial gene name for Inpp5d (SHIP) used in this thesis</td>
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<tr>
<td>SHPS-1</td>
<td>SH2 domain containing protein tyrosine phosphatase substrate-1</td>
</tr>
<tr>
<td>Sirpα</td>
<td>signal regulatory protein alpha</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SR</td>
<td>scavenger receptor</td>
</tr>
<tr>
<td>ST2</td>
<td>suppressor of tumorigenicity 2</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>sTLR</td>
<td>soluble decoy TLR</td>
</tr>
<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>TAA</td>
<td>tumour associated antigen</td>
</tr>
<tr>
<td>TAB2</td>
<td>TAK1-binding protein 2</td>
</tr>
<tr>
<td>TAK1</td>
<td>transforming-growth-factor-β-activated kinase 1</td>
</tr>
<tr>
<td>TBK1</td>
<td>TRAF-family member associated NFκB activator binding kinase 1</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>helper T cell</td>
</tr>
<tr>
<td>Tip DC</td>
<td>TNFα and iNOS producing dendritic cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Tollip</td>
<td>Toll-interacting protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumour necrosis-factor-receptor associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adapter molecule (also known as TICAM2)</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter protein inducing IFNβ (also known as TICAM1)</td>
</tr>
<tr>
<td>UBC13</td>
<td>ubiquitin-conjugating enzyme 13</td>
</tr>
<tr>
<td>UEV1A</td>
<td>ubiquitin-conjugating enzyme E2 variant 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>X</td>
<td>any amino acid</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>β-ME</td>
<td>beta-mercaptoethanol (2-mercaptoethanol)</td>
</tr>
</tbody>
</table>
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Co-Authorship Statement

All experiments, with the exception of those described below, were performed by Frann Antignano. I conceived of and designed all of the experiments contained within this thesis. I analyzed and interpreted all of the data, wrote and edited the dissertation.

In Chapter 3, the RT-PCR was performed by Angela Zhang (Terry Fox Lab) under the supervision of Jens Ruschmann (Terry Fox Lab). In addition, several of the experiments were performed either by or with the assistance of co-operative education students under my supervision, including Mariko Ibaraki and Connie Kim.

In Chapter 4, the RT-PCR was performed by Jens Ruschmann (Terry Fox Lab). Again, several of the experiments were performed either by or with the assistance of co-op students Mariko Ibaraki, Connie Kim and Julienne Jagdeo.

Research in Chapter 5 was performed with the help of Drs. Michael Hughes and Jami Bennett (Biomedical Research Centre).

In Chapter 6 the qPCR was performed by Dr. Carla Cohen (Terry Fox lab). As well, although not directly contributing to any specific figure, Melisa Hamilton participated in the generation of experimental ideas.

Dr Gerald Krystal interpreted data and edited the thesis.
CHAPTER 1: INTRODUCTION

1.1 Hematopoiesis

Blood contains many different kinds of mature cells, each with a unique, finite life span and specialized function. Through the process of hematopoiesis, these short-lived (primarily) circulating cells are replaced by newly differentiated progenitors, resulting in the maintenance of a relatively constant number of each cell type (Smith, 2003). Early studies investigating the effects of chemical agents and ionizing radiation on mouse blood and bone marrow demonstrated that death from a lethal dose of X-rays could be prevented by shielding the spleen or by injecting spleen cells from a non-irradiated mouse (Jacobson and Marks, 1949). Although initially attributed to unknown humoral factors, further study revealed that cells of the spleen or bone marrow (BM) were in fact responsible for this protection (Jacobson et al., 1951; Lorenz et al., 1951). Subsequent animal studies revealed that bone marrow contains transplantable cells that are capable of reconstituting the entire hematopoietic system of a lethally irradiated recipient (Ford et al., 1956; Main and Prehn, 1955; Nowell et al., 1956).

In 1961, Till and McCulloch demonstrated that a subset of bone marrow cells could form macroscopic colonies in the spleen when injected into a lethally irradiated mouse (Till and McCulloch, 1961). These colonies, termed colony forming unit spleen (CFU-S), contained progeny of multiple blood lineages and a subset of these colonies were capable of reforming colonies and reconstituting hematopoiesis in secondary irradiated recipients (Wu et al., 1967). The cells giving rise to these colonies thus displayed some of the hallmarks of hematopoietic stem cells (HSCs): they were capable of self-renewal and they could differentiate into mature, phenotypically recognizable blood cells. It was, however, later determined that these CFU-S forming cells were actually slightly more differentiated since they were not capable of long term engraftment and therefore not true HSCs (Schofield, 1978). To be defined as an HSC a cell must be a primitive hematopoietic cell capable of reconstituting both myelo- and lymphopoiesis for the lifetime of the recipient (Osawa et al., 1996).
In mouse bone marrow, HSCs have been identified in a small population of cells, termed LSK cells, which lack lineage specific markers (lin⁻) but express high Sca-1 (a cell surface glycosylphosphatidylinositol (GPI)-linked adhesion protein) and c-kit (the steel factor receptor) (Akashi et al., 2005; Morrison and Weissman, 1994; Morrison et al., 1995; Spangrude et al., 1988). LSK cells can be further subdivided into long-term (Thy-1\textsuperscript{low}Flt3⁻) and short-term repopulating HSCs (Thy-1\textsuperscript{low}Flt3⁺) and multipotent progenitors (MPPs, Thy-1⁻Flt3⁺) (Christensen and Weissman, 2001). These compartments are succeeded by progressively more lineage-restricted and differentiated progeny with diminishing abilities to self renew (reviewed in (Bryder et al., 2006)). Under normal homeostatic conditions, the HSC population remains relatively constant since individual HSCs divide to produce one daughter cell that self-renews and returns to a state of quiescence (Bradford et al., 1997) and one that proceeds down a differentiation pathway to give rise to specialized mature blood cells. In the absence of stresses, the rate of mature cell output is tightly regulated by intrinsic and extrinsic factors (Barreda et al., 2004) to ensure that cell survival, proliferation and differentiation is counter-balanced by apoptosis of progenitors and death of mature cells (Domen et al., 2000). Stresses, such as injury, hypoxia and infection result in increased hematopoietic cell production within specific lineages (Rogowski et al., 1998).

HSCs were the first tissue-specific stem cell to be isolated (Spangrude et al., 1988) and remain the only stem cell in frequent clinical use. In particular, HSCs have been used in the treatment of leukemias and autoimmune diseases (Weissman, 2000). Despite intensive research to fully understand HSC biology, much knowledge is lacking. In particular, the heterogeneity of the LSK compartment has become increasingly apparent and recent studies have shown that, based on white blood cell repopulation patterns, HSCs can be divided into four functionally distinct subsets (Dykstra et al., 2007). As studies and technology advance, HSC therapy may be tailored more specifically to the disease being treated.
1.2 Hematopoietic cascade

The human hematopoietic system produces an estimated 1.5 million blood cells every second in order to offset the very short lifespan of most effector cells (reviewed in (Bryder et al., 2006)). It is well established that this process is mediated by a rare population of pluripotent HSCs which are uniquely able to self renew as well as to differentiate and commit to all blood cell lineages (Reya et al., 2001). As HSCs differentiate there is increasing lineage restriction and amplification until finally functionally specialized mature cells are produced (Cantor and Orkin, 2001). Once thought to be well elucidated, the roadmap from the HSC compartment to terminally differentiated cells has recently been debated and is under revision (Graf, 2008).

The previously accepted 'classical' scheme of hematopoietic differentiation is that proposed by Akashi, Kondo and Weissman (Fig 1.1) (Kondo et al., 1997). This scheme consists of a lineage tree composed primarily of binary decisions where a long term HSC gives rise to a short-term HSC and then a MPP. After this point the restrictive nature of the cells increases and the next lineage commitment step is a bifurcation into the common lymphoid progenitor (CLP) (Kondo et al., 1997) and a common myeloid progenitor (CMP) (Akashi et al., 2000). Evidence for this scheme has been provided by gene transcription profiles comparing CMPs and CLPs (Miyamoto et al., 2002) which suggest that the myeloid versus lymphoid decision is made at this stage and thus, in this scheme of hematopoiesis, CLPs and CMPs produce mutually exclusive cells. CLPs give rise to pro-B and pro-T cells which further differentiate into mature B and T cells. As well, CLPs differentiate to produce natural killer (NK) (Kondo et al., 1997) and NKT cells which branch off of the T cell lineage (Kronenberg, 2005). On the other side of this lineage tree, CMPs generate the more restricted granulocyte-macrophage progenitor (GMP), which produces macrophages (MΦ) and granulocytes, and the megakaryocyte-erythrocyte progenitor (MEP) that gives rise to megakaryocytes and erythrocytes (Akashi et al., 2000). The granulocyte compartment includes neutrophils, eosinophils (Denburg et al., 1985), mast cells and basophils (Akashi et al., 2005; Arinobu et al., 2005).
Figure 1.1 Classical model of hematopoiesis. Pluripotent HSCs give rise to all mature blood cell types. Following the HSC compartment, there is a bifurcation into CLPs or CMPs which give rise to increasingly lineage-commited cells which ultimately develop into terminally differentiated cells. Adapted from Kondo et al (Kondo et al., 1997) and other references in section 1.2.
In contrast to the hematopoietic model described above, newer data have revealed that alternative developmental pathways also exist to generate myeloid and lymphoid cells. Included in these recent findings are the description of a macrophage/T cell/B cell progenitor which lacks megakaryocyte and erythroid potential (Adolfsson et al., 2005). This suggests that MΦs can be generated by two pathways, one that originates from the classical CMP and another coming from a lympho-monocytic progenitor. Other recent studies using single cell assays have found that CD4/CD8 double negative (DN1) early T cell progenitors (ETPs), as well as DN2 cells, lack B cell potential but retain myeloid potential (Bell and Bhandoola, 2008; Wada et al., 2008). From the DN3 stage of T cell development onward, precursors are no longer capable of myeloid differentiation. The myeloid cells produced via this pathway appear to be primarily MΦs, however, some granulocytes (Bell and Bhandoola, 2008), dendritic cells (DCs) and NK cells (Wada et al., 2008) have also been observed.

DCs (to be discussed in greater detail later) can be broadly classified as either conventional/myeloid DCs (CD11c⁺CD11b⁺) or lymphoid DCs (CD8α⁺). However, specialized subsets also exist including plasmacytoid DCs as well as Langerhans cells (LCs) found in the skin (reviewed in (Shortman and Liu, 2002)). Both myeloid and lymphoid DCs can be generated from either CMPs and CLPs and are functionally and phenotypically indistinguishable (Manz et al., 2001a; Manz et al., 2001b; Traver et al., 2000), whereas B cell precursors (pro-B cells) and MEPs are incapable of producing DCs (Manz et al., 2001b).

1.3 A Brief introduction to the immune system

When a pathogen enters the body the immune system must respond and promptly eliminate it to prevent its replication, spread and harm to the host. Vertebrates are endowed with two arms of immunity: the innate and adaptive immune systems (Cooper, 2008). The more ancient innate immune system is responsible for immediate control of a pathogen infection. This includes the natural barriers of the body such as the skin and mucosa as well as the white blood cells including granulocytes, MΦ, mast cells and NK
cells and the anti-microbial products they produce. Pathogens have co-evolved with their hosts and have adapted mechanisms to evade the innate immune system, hence the need for the adaptive immune system. The adaptive immune system is comprised of the other white blood cells, B and T lymphocytes. B cells exert their effector function through the production of antibodies directed against the pathogen in question, while T cells can either help (i.e., CD4+ helper T cells) to activate other effector cells or they can directly kill infected cells (CD8+ cytotoxic T cells (CTL)) (Janeway et al., 2005). Both B and T cells possess receptors (B cell receptors (BCRs) and T cell receptors (TCRs), respectively) that undergo a semi-random generation to develop potentially infinite variation. This random generation, however, does produce, on occasion, receptors specific against the body's own tissues. As a result, the process of tolerance exists to eliminate potentially self-reactive B and T cells either before they enter the circulation (central tolerance) or after (peripheral tolerance). For B cells, central tolerance occurs primarily in the bone marrow (Nossal, 1994) while T cell central tolerance occurs in the thymus (Gallegos and Bevan, 2006), preventing the production of self-destructive lymphocytes.

The innate and adaptive immune systems often cooperate to eliminate certain pathogens, with the innate immune system directing the adaptive immune response (Lee and Iwasaki, 2007). In the periphery, naïve B and T cells (lymphocytes that have not encountered an antigen (Ag)) require signals through their BCR and TCR, respectively, in order to become active. The clonal expansion of antigen-specific lymphocytes requires the recognition of foreign Ag in the context of a danger signal (Matzinger, 1998). Notably, the frequency of lymphocytes expressing a particular Ag-specific receptor is extremely low (e.g., a T cell with a particular TCR is approximately 1 in 2x10^5 (Blattman et al., 2002)). As such, lymphocytes cannot simply circulate through the body in hopes of finding their specific Ag. As a result, the body has concentrated lymphocytes in lymphoid organs that include the lymphatic vessels, spleen, lymph nodes and Peyer's patches. This concentration results in the need for a separate group of cells, the Ag presenting cells (APCs), which circulate throughout the body and specialize in Ag uptake and processing and recognizing infectious organisms in the periphery, after which they
travel to a lymphoid organ to deliver an antigenic signal to cognate lymphocytes (Villadangos and Schnorrer, 2007). Because of the concentration of lymphocytes in the lymphoid organs, the chance of the pathogen specific Ag-bearing APC encountering the right lymphocyte is increased substantially.

DCs are the most potent T cell activating APC in the body. DCs capture, process and present both self and foreign antigenic protein in the form of peptides (Villadangos and Schnorrer, 2007). These peptides are presented bound in the groove of the major histocompatibility complex (MHC) on the surface of the DC. For the most part, intracellular-derived peptides are presented on MHC class I (MHCI) molecules and are recognized by the TCR of cognate CD8+ T cells, while exogenous peptides are presented on MHC class II (MHCII) for interaction with the TCR of cognate CD4+ T cells (Cresswell, 2005). MHCI molecules are present on all cells, but only DCs, B cells, MΦs and some monocytes express MHCII (Bryant and Ploegh, 2004). These cells also express co-stimulatory molecules such as CD80/CD86 and CD40, required for full T cell activation (Greenwald et al., 2005) and are referred to as professional APCs.

Priming of naïve T cells occurs only when TCR-MHC/peptide interaction is coupled with a second, co-stimulatory, signal provided by interactions of CD80/CD86 on APCs with CD28 on T cells (Cools et al., 2007). The expression of high levels of co-stimulatory molecules on APCs occurs only in the presence of microbial stimulation. Thus, in the steady state when DCs are sampling and presenting self-peptides, they will be unable to induce a T cell response. Rather, they will induce death or anergy in T cells that recognize self-peptides (Cools et al., 2007). Importantly, this system of tolerance is not without error and thus not all self-reactive T cells are eliminated. When DCs are stimulated by danger signals in conjunction with Ag uptake, they mature and substantially increase the surface expression of MHCII/peptide and co-stimulatory molecules (Lee and Iwasaki, 2007). In addition, DCs activated in this way will secrete cytokines important for directing T cell responses.
In summary, DCs are capable, depending on the situation, of inducing T cell tolerance or T cell activation and drive the induction of an adaptive immune response. DCs are crucial for the regulation of the immune system against pathogen invasion as well as for protection from non-specific or self-antigen activation of T cell functions.

1.4 Dendritic cell discovery

Although DCs were not discovered and characterized until much later, the initiating steps to their discovery began in 1882 when Russian biologist (and the winner of the first Nobel Prize in cellular immunology in 1908) Illya Metchnikoff identified the MΦ (Tauber, 2003). Metchnikoff had observed the presence of amoeboid digestive cells, which he termed phagocytes from the Greek ‘phagos’ (to eat) and ‘cyte’ (cell). He theorized that these cells were likely also present in vertebrates and may be responsible for ingesting and removing disease causing microbes.

In 1966 red blood cells from sheep were added to a suspension of mouse spleen cells, generating the first in vitro primary antibody response (Mishell and Dutton, 1966). By separating spleen cells into two populations based on their ability to adhere, or not, to dishes, it was later reported that this antibody (Ab) response required the presence of adherent accessory cells present in the spleen, of which a large component were MΦs (Mosier, 1967). Exposure of adherent cells to Ag for even brief periods was sufficient to induce an antibody response (Mosier, 1967). Initial assumptions were that MΦs were likely the APC responsible for this effect and it wasn’t until 1973 that the first paper appeared describing a new splenic adherent cell type found amongst the MΦs (Steinman and Cohn, 1973). In this paper a novel adherent cell type, identified by phase and electron microscopy, cytochemistry and cinematography, was reported to be present within the peripheral organs of mice (spleen, lymph nodes and Peyer's patches). These constituted only 0.1-1.6% of nucleated cells and had a very distinctive morphology, including a very large, refractile nucleus containing small nucleoli, many mitochondria and an abundant cytoplasm arranged in processes of varying lengths and widths (Steinman and Cohn, 1973). Although they displayed characteristic movements they did
not appear to actively endocytose. As a result of their unique morphology and long projections, Steinman and Cohn dubbed these cells “dendritic cells” derived from the Greek word “dendreon” for tree. As touching evidence of scientific romance, it is said that Steinman’s first impulse was to name them “claudiocytes” after his wife because of the cells’ long slender projections and graceful movements.

When \textit{in vitro} functional tests began with DCs it became even more clear that they were in fact a \textit{bone fide} novel cell type, ie., it was determined that they did not represent morphological variants of either MΦs or lymphocytes since they did not have the endocytic capacities of MΦs nor did they express lymphocyte surface differentiation markers (Steinman and Cohn, 1974). In addition, they did not synthesize collagen-like macromolecules, nor were they capable of serving as stem cells for erythroid or myeloid colony formation and they did not retain Ags or immune complexes on their surface (Steinman and Cohn, 1974).

Early \textit{in situ} studies revealed several more novel features of DCs in their natural environment. Splenic DCs, for example, were found to have a low proliferation rate, with a turnover rate of 10% per day to keep total cell numbers relatively constant, even during an immune response (Steinman et al., 1974). DCs appeared to be derived from a group of precursor cells in which at least three cell divisions were required for differentiation and at least some of these precursors existed in the bone marrow in a different morphological state than their end product (Steinman et al., 1974). In addition, several more features were identified that further distinguished DCs from MΦs. DCs were only found in the spleens of mice that were at least 3-4 weeks of age whereas neonate spleens contained only MΦs (Steinman et al., 1974). As well, MΦs in the spleen (Steinman et al., 1974) and the peritoneal cavity (Thompson and van Furth, 1970) could withstand low doses of steroids and ionizing radiation while DCs were lost under these conditions (Steinman et al., 1974).

Immune reaction studies soon revealed the functions of DCs. For example, within two days following administration of sheep red blood cells to a mouse spleen, DCs with
an even more irregular shape than their naïve counterparts appeared in large numbers in
the proximity of developing plasma cell responses (Steinman et al., 1975). A major
discovery paving the way to understanding DC functions was the characterization of the
proteins expressed on the surface of DCs. Like B cells, DCs were found to express high
levels of MHCs, but lacked surface immunoglobulin (Ig) (Steinman et al., 1979). Using a
mixed leukocyte reaction (MLR), a technique designed to induce T cell proliferation in
response to allo-antigens, Steinman found that DCs strongly induced T cell proliferation
(Steinman and Witmer, 1978). In fact, they found DCs were at least 100 times more
potent than bulk spleen cells in stimulating an MLR reaction. This result suggested that
DCs, and not MΦs, were the critical accessory cell required for the initiation of many
immune responses (Steinman and Witmer, 1978).

1.5 Phenotypic and functional diversity of mouse DC subsets

Since the initial discovery of DCs, investigators have become aware that DCs are
not a single homogenous cell type but are composed of several subsets with specialized
functions (Shortman and Liu, 2002). Discovery of this heterogeneity was aided in large
part by advancements in flow cytometry and hybridoma technology which allowed for
more advanced surface molecule characterization (Crowley et al., 1989; Vremec et al.,

The dendritic cell literature professes several different subset classification
systems as well as nomenclatural differences in terms of the surface marker expression
phenotypes that constitute particular DC subsets. In addition, these classification systems
are further complicated by considering the activation state -as a steady state DC and an
inflammatory DC can vary in their phenotype and lead to (mis)classification into unique
subsets. In general, DCs can be broadly classified as either conventional DCs (cDCs),
sometimes referred to as myeloid DCs (mDCs), or lymphoid DCs. DCs can be found in
non-lymphoid tissues, in the circulation or in lymphoid tissues (Jacobs et al., 2008).
Alternatively, DC subsets can be elaborately classified based on whether they are
precursor DCs (pre-DCs), which require further differentiation to achieve full dendritic
form and function, or, as fully mature DCs; whether they are migratory in nature, traveling from a tissue back into the lymphatic system (migratory), or if they already reside within lymphoid organs (resident) or develop in the bone marrow and circulate in the blood (circulating); or whether they are in a resting or activated state (following an infection or other inflammatory stimulus) (Naik, 2008). For the sake of clarity of presentation, I have attempted to provide a comprehensive yet simple classification of DC subsets (Table 1.1).

1.5.1 Pre-DCs

The pre-DC subset is, in fact, not a DC at all but rather a cell on its way to becoming a DC. Pre-DCs are the immediate precursor to a DC and can develop into a DC in the steady state or as the result of stimulation (often infection or inflammation) (Shortman and Naik, 2007). In addition to their DC potential, some pre-DCs also have unique functions of their own. One example of a cell that can be classified as a pre-DC is the monocyte (Naik, 2008). Monocytes are phagocytic cells found in blood, bone marrow and at low levels in the spleen and are precursors to both MΦs and DCs (Hume et al., 2002). In response to macrophage colony stimulating factor (M-CSF), monocytes will differentiate \textit{in vitro} into MΦs (Becker et al., 1987) while in response to granulocyte-macrophage colony stimulating factor (GM-CSF) they differentiate into DCs (Leon et al., 2004).

1.5.2 Plasmacytoid DCs

Another type of steady state pre-DC is the pDC which, like the monocyte, has features of its own (O'Keeffe et al., 2003). In mice pDCs can be found in the blood (O'Keeffe et al., 2003), thymus (Okada et al., 2003), bone marrow (Nikolic et al., 2002), liver (Lian et al., 2003; Pillarisetty et al., 2004) and lymphoid organs (spleen and lymph nodes) (Nakano et al., 2001; Nikolic et al., 2002). They appear as relatively long-lived round plasma-like cells without typical dendritic protrusions but, upon microbial activation, differentiate into a more conventional DC (O'Keeffe et al., 2002).
Table 1.1 Summary of the features and tissue distribution of major mouse DC subsets

<table>
<thead>
<tr>
<th>Major DC subset</th>
<th>CD8α+ CD11b-</th>
<th>CD8α- CD11b+</th>
<th>Plasmacytoid (pDC)</th>
<th>CD8α- CD11b-</th>
<th>Langerhans</th>
<th>Dermal</th>
<th>Monocyte derived (inflammation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>CD11cα- CD8α+ CD11b- DEC205+ CD4-</td>
<td>CD11cβ- CD8α+ CD11b- DEC205+ CD4+</td>
<td>CD11cα- CD8α- CD11b+ B220+ Gr1+</td>
<td>CD11cβ- CD8α- CD11b- DEC205+ CD4-</td>
<td>CD11cα- CD8α+ CD11b+ DEC205+ Langerin+</td>
<td>CD11cα- CD8α- CD11b+ DEC205+ CD4-</td>
<td></td>
</tr>
<tr>
<td>Organ</td>
<td>Spleen, LN, PP, LP, MLN, Thymus</td>
<td>Spleen, LN, PP, LP, MLN</td>
<td>Spleen, LN, PP, LP, MLN</td>
<td>Spleen, LN, PP, LP, MLN</td>
<td>Epithelium of skin, LN, MLN</td>
<td>Dermis of skin, LN, MLN</td>
<td>Inflamed spleen, LN</td>
</tr>
<tr>
<td>Cytokines</td>
<td>High IL-12 Low IL-10</td>
<td>Low IL-12 High IL-10</td>
<td>Type I IFNs</td>
<td>High IL-12 Low IL-10</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>T-helper response</td>
<td>T_h1</td>
<td>T_h2 (except LP where T_h17)</td>
<td>T_h1 or T_reg</td>
<td>T_h1</td>
<td>T_h1</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Cross-presentation</td>
<td>++++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>TLR expression</td>
<td>TLR1,2,3,4,6,8,9</td>
<td>TLR1,2,4,5,6,7,8,9</td>
<td>TLR1,2,4,5,6,7,8,9</td>
<td>TLR1,2,3,4,5,6,7,8,9</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

LN- lymph node, LP- lamina propria, MLN- mesenteric lymph node, PP- Peyer's patch
Adapted from Pulendran et al (Pulendran et al., 2008) and Iwaskai and Medzhitov (Iwasaki and Medzhitov, 2004).

Phenotypically, pDCs can be identified in all organs by the expression of CD11c (integrin α x), B220, Gr-1 and MHCIId (CD11c<sup>intermediate</sup>CD8α<sup>+</sup>CD11b<sup>B220<sup>-</sup>Gr<sup>-1<sup>+</sup>MHCIId<sub>low</sub></sup> (Naik, 2008; Pulendran et al., 2008). In mouse blood, pDCs are CD4<sup>-</sup>CD8<sup>+</sup>. However, in all other organs they can be found with any of the following phenotypes CD4<sup>-</sup>CD8<sup>+</sup>, CD4<sup>-</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup> (Hochrein and O'Keeffe, 2008). CD4 is a differentiation marker, where CD4<sup>-</sup> pDC differentiate into CD4<sup>+</sup> pDCs (O'Keeffe et al., 2002). Another differentially expressed molecule on pDCs is Ly49Q, which is not only
differentially expressed on pDC subsets, but also among mouse strains (Hochrein and O'Keeffe, 2008).

The primary function of pDCs is immune surveillance of pathogens. In response to viral (Siegal et al., 1999) and bacterial (O'Keeffe et al., 2003) stimulation they secrete chemokines and cytokines, in particular Type I interferon (IFN-α/β) (Asselin-Paturel et al., 2001). This makes pDCs particular important for the clearance of viral infections.

1.5.3 Conventional DCs

Conventional DCs (cDCs) are fully differentiated DCs that reside in lymphoid tissues and are present in all lymphoid organs of the mouse in an immature state (Wilson et al., 2003). In the spleen, resident DCs can be identified by the expression of CD11c and intermediate expression of MHCII, and then further subdivided by the expression of CD4 and CD8 (Vremec et al., 1992; Vremec et al., 2000). Approximately 25% of the total cDCs in the spleen are CD8⁺CD4⁻ resident DCs which, in the steady state, are found in the T cell areas of the spleen architecture (Kamath et al., 2000). In immune responses, CD8⁺ DCs are considered extremely important due to their ability to secrete large amounts of the bioactive form of the proinflammatory cytokine interleukin 12 (IL-12p70) (Hochrein et al., 2001), resulting in the induction of a Th1 response (Maldonado-Lopez et al., 1999a; Maldonado-Lopez et al., 1999b). In addition, CD8⁺ DCs also secrete IL-6, TNFα and low levels of the chemokines MIP3α/β and RANTES upon activation (Proietto et al., 2004).

A key feature that distinguishes CD8α⁺ cDCs is their ability to ‘cross-present’ Ags, i.e., they can present exogenous Ag very efficiently on MHCII, allowing for activation of CD8⁺ T cells (den Haan et al., 2000; Pooley et al., 2001; Schnorrer et al., 2006). This function is important in the context of viral infections, tumour immune surveillance and maintenance of peripheral tolerance (Hawiger et al., 2001; Kurts et al., 1997).
Resident CD8+ cDCs can be divided into CD4+ and CD4- . The CD8'-CD4+ cDCs comprise approximately 50% of the splenic cDCs and are localized to the marginal non-T cell zones of the spleen (De Smedt et al., 1996). These cells produce high levels of the inflammatory chemokines MIP3α/β and RANTES (Proietto et al., 2004) both in a resting state and in response to activation. Unlike, the CD8+ subset, the CD8'-CD4+ subset have very little, if any, ability to cross-present (den Haan and Bevan, 2002; Pooley et al., 2001) but are very potent MHCII-dependent activators of CD4+ T cells and have been found to induce a Th2 response (Hammad et al., 2004).

The remaining 25% of splenic cDCs are negative for both CD8 and CD4 and have been found to very closely resemble the CD8'CD4+ DCs in function. Like their CD4+ counterparts, CD4- DCs also produce high levels of MIP3α/β and RANTES. However, this production is primarily in response to stimulation and does not occur under steady state conditions (Proietto et al., 2004). Also, CD8'CD4- cDCs are poor cross-presenters but very efficient in direct MHC-I and MHC-II presentation and T cell activation (Schnorrer et al., 2006).

Other resident cDCs can be found in the lymph nodes (LNs) and thymus. LNs contain resident CD8+ and CD8- cDCs which express CD11c and low levels of MHCII, much like in the spleen (Wan and Dupasquier, 2005). LN CD8+ (MHCII<sub>low</sub>CD11b-CD205+B220-) are the major producers of IL-12p70 in the nodes, but the amount is much reduced compared to their splenic counterparts (Hochrein et al., 2001). Also, like those found in the spleen, LN CD8α+ cDCs are very good at cross-presentation. CD8- cDCs found in LNs can be found as either CD4+ or CD4-, but in both cases they are also CD11b+CD205'B220'. However, the expression of CD4 is somewhat controversial since some studies suggest that CD4 expression may be acquired from sampling CD4+ T cells (Henri et al., 2001).

In the thymus, DCs are located in the medulla or at the cortico-medullary junction where they may play a role in negative selection of medullary thymocytes, induction of central tolerance (Wu and Shortman, 2005) and the positive selection of medullary
regulatory T cells (Watanabe et al., 2005). Thymic DCs are CD11c+, and they are also positive for CD8αβ. However, some are CD8α\textsubscript{high} and some CD8α\textsubscript{low}. These two groups differentially express signal regulatory protein alpha (Sirpα, a regulator of phagocytosis in DCs), where CD8α\textsubscript{high} are negative for Sirpα and CD8α\textsubscript{low} are Sirpα+ (Lahoud et al., 2006). All thymic DCs, regardless of CD8 expression, are also CD205+ and CD11b\textsubscript{low} (Vremec et al., 2000).

### 1.5.4 Migratory DCs

Migratory DCs are the prototypical DCs that are found in the periphery where they act as sentinels of the immune system and migrate via lymphatic vessels to draining lymphoid organs. This migration occurs at a basal rate under steady state conditions (Hemmi et al., 2001; Jakob et al., 2001) but is enhanced upon innate activation and these DCs are found in draining lymphoid organs in a mature state (Wilson et al., 2003). This allows for the distinction of resident MHCII\textsubscript{low} from MHCII\textsubscript{high} migratory DCs (Henri et al., 2001). Langerhans cells (LCs) of the epidermis and dermal-derived DCs fall into the resident DC category.

LCs and dermal DCs within subcutaneous LNs are relatively long-lived cells (Kamath et al., 2002) that make up 2-4% of the total cellularity in the epidermis (Naik, 2008). LCs (CD11c\textsuperscript{high}CD8\textsuperscript{low}CD205\textsuperscript{high}) express the surface marker C-type lectin, Langerin, and can be recognized by the presence of intracellular Birbeck granules. Dermal-derived DCs, on the other hand, are CD11c\textsuperscript{high}CD8CD11b\textsuperscript{+}CD205\textsuperscript{+} (Kamath et al., 2002). Skin LCs and dermal-derived DCs are continuously sampling their environment (Wilson et al., 2003) and, upon entry into the lymphatic system, become activated regardless of whether they are in a steady or an inflammatory state. Although it has been suggested that these cells provide T cell priming in response to cutaneous infections definitive evidence to support this hypothesis remains elusive. The specific role LCs play in T cell activation in LNs remains controversial and may depend on the nature of the Ag or the context in which the Ag is taken up (ie., if the DC itself is infected.
with a virus, or the kind of innate immune activation that the DC has received) (Kissenpfennig and Malissen, 2006).

Migratory DCs can also be found in non-skin draining LNs and are heterogeneous in nature. For example, the mediastinal LN which drains the lungs contains a migratory DC subset that is F4/80\(^+\)CD11b\(^-\). Cells of a similar phenotype can also be found in LNs that drain the kidneys and liver while skin and mesenteric LNs do not contain any DCs of a similar phenotype (Belz et al., 2004).

Of note, mice have very few cells in the blood or BM that display the characteristic DC markers CD11c\(^{\text{high}}\) and MHCII. Rather, the cells (DCs) found in the blood (O'Keeffe et al., 2003) and BM (Diao et al., 2006; Hochrein and O'Keeffe, 2008) are CD11c\(^{\text{int}}\)MHC\(^{\text{low}}\)-CD11b\(^+\)CD4\(^-\)CD8\(^-\). The few CD11c\(^{\text{high}}\) cells in mouse peripheral blood are likely B220\(^+\) pDCs, DX5\(^+\) NK cells, pre-DCs or monocytes (O'Keeffe et al., 2003).

In addition to those DCs already described above, there also exist non-lymphoid organ specific DCs that have specific surface phenotypes and functions. As well, there are DCs that are induced as the result of specific infections such as the tumour necrosis factor-\(\alpha\) (TNF\(\alpha\)) and inducible nitric oxide (iNOS) producing DC (Tip DC) which have a unique CD11c\(^{\text{int}}\)CD11b\(^{\text{high}}\)Mac-3\(^+\) phenotype. Tip DCs were first identified in the spleen of mice 1-2 days after infection with *Listeria monocytogenes* (Serbina et al., 2003a).

### 1.6 Dendritic cell ontogeny

The majority of DC characterization has been performed with cells from adult mice. However, phenotypic and functionally unique DCs have been identified in embryonic and neonatal mice. Although DC populations were initially described as being absent from newborn mice and only reaching adult concentrations at 3-4 weeks of age (Steinman et al., 1974), recent work has shown that during mouse ontogeny the composition of DC populations differs from that of adult mice.
It is well established that neonates are incapable of mounting a full immune response to infection and are more susceptible than adults to Ag-induced tolerance (Adkins, 2000; Billingham et al., 1953). Early immune responses in neonates are generally T\textsubscript{H}2 skewed and result in poor cytotoxic T cell responses, and increased susceptibility to intracellular pathogens (Adkins, 2000). Initial studies to determine the reason for this focused primarily on T cells and their qualitative and quantitative differences in neonates versus adult mice. However, in spite of the neonate T cell differences, it has been found that under appropriate stimulatory conditions neonatal T cells could mount adult-like T cell responses \textit{in vitro} (Adkins, 1999) and T\textsubscript{H}1 responses could be induced \textit{in vivo} (Forsthuber et al., 1996). In addition, the idea that neonatal T cells were more susceptible to the induction of tolerance was questioned as it became clear that it was the nature of APC that determined T cell fate.

DC development appears to first occur around embryonic day 17 (E17) when a population of CD11c\textsuperscript{+}B220\textsuperscript{-} DCs and CD11c\textsuperscript{int}B220\textsuperscript{+} pre-DCs can be observed at very low frequency in the thymus (Dakic et al., 2004). Although initially thought to be absent in the spleen until two weeks of age (Lu et al., 1980) more recent studies suggest that DCs become detectable in the spleen at birth (Dakic et al., 2004; Sun et al., 2003). DCs from neonatal mice express very low levels of MHCII, costimulatory markers CD80/CD86 and CD11c but high levels of monocyte markers F4/80 and CD11b, as well as the granulocyte marker Ly6G. These are all signs of an immature DC phenotype (Muthukkumar et al., 2000). These cells have a limited ability to present Ag and activate T cells but, as the mice age, the expression of DC markers (MHCII, CD80/86 and CD11c) increases as does their ability to present Ag (Muthukkumar et al., 2000).

The DC composition of neonatal mice is also significantly different from that of adult mice. At birth only two DC subsets can be found in the spleen: pDCs (CD11c\textsuperscript{low}B220\textsuperscript{+}) and CD11c\textsuperscript{+}CD4\textsuperscript{-}CD8\textsuperscript{-} cDCs (Sun et al., 2003). In the following week CD8\textsuperscript{+} DCs develop and expand to adult levels (Sun et al., 2003) which likely account for the CTL responses that can be generated by CD11c\textsuperscript{+} splenic DCs at day seven. The
CD4+ DC compartment develops more slowly but becomes the most predominant by three weeks of age (Sun et al., 2003).

Although there is now a consensus about DC composition during mouse ontogeny, their functional ability appears to still be contested. Some groups find neonatal DCs have a reduced capacity to produce IL-12p70 (Dakic et al., 2004) while others find IL-12p70 production by DCs to be particularly high after birth (Sun et al., 2003). It is possible that the particular DC subset each group is isolating is different and accounts for the disparity in their results.

It appears that it isn't until approximately four weeks of age that mice acquire their full complement of completely functional DCs (Dakic et al., 2004; Sun et al., 2003). This could account for the relative incompetence of the neonatal immune system as suggested by studies showing that neonatal mice treated with fms-like tyrosine kinase 3 ligand (Flt3L) (which increases DC numbers) have enhanced immunity to intracellular pathogens and viral infections (Vollstedt et al., 2003). Thus, it seems a complete DC system is required for the effective initiation of an adaptive immune response.

1.7 Regulation of dendritic cell differentiation: growth factors, cytokines and transcription factors

As hematopoietic cells, DCs ultimately originate from HSCs. From this point on, however, the differentiation of DCs becomes increasingly complex as there are multiple subtypes, maturation states and tissue distributions, all with potentially unique differentiation pathways. As mentioned earlier, the first step downstream of the HSC is the bifurcation into the CMP and the CLP. Although it was initially thought that DCs originate solely from the CMP because of their similarity to macrophages, this was quickly disproved with the finding that lymphoid restricted precursors in the thymus could generate DCs and in fact bone marrow isolated CLP and CMP could both give rise to pDCs and cDCs in vivo (Manz et al., 2001a; Manz et al., 2001b; Traver et al., 2000). Further subdivision of CMPs and CLPs revealed that expression of the receptor fms-like
tyrosine kinase 3 (Flt3) on the surface of progenitors conferred upon them enhanced DC potential such that only the Flt3\(^+\) cells (which constitutes 30% and 70% of the CMP and CLPs, respectively) are able to generate all cDC and pDC lineages when transplanted \textit{in vivo} (Karsunky et al., 2003). DCs are the only cell type definitively shown to have both a myeloid and lymphoid origin (Fig 1.2).

As differentiation proceeds from these very early progenitors to late progenitors, the precise steps involved become less clear. Studies looking for intermediate DC precursors have used bone marrow cells that are lin\(^-\)CD11c\(^-\)MHCII\(^-\) and still express early hematopoietic precursor markers such as CD117 (Fogg et al., 2006). Within this compartment CX3CR1\(^+\) cells were selected and found \textit{in vitro} to be a common precursor of DCs and macrophages, but were incapable of granulocyte production (Fogg et al., 2006). Upon transfer into mice, these cells produced both CD8\(^+\) and CD8\(^-\) cDCs, but did not generate pDCs (Fogg et al., 2006). This result indicated that cDC and macrophage potential are retained longer than pDC potential, which branches off earlier during development. Further evidence of this earlier branch point came from studies looking at later DC progenitors which already expressed CD11c but lacked MHCII expression. These studies revealed that CD11c\(^+\)B220\(^-\) precursors have the capacity to produce pDCs, CD8\(^+\) and CD8\(^-\) cDCs, but CD11c\(^+\)B220\(^-\) precursors cannot generate pDCs (Diao et al., 2004). Even more immediate precursors have been identified for specific DC subsets, including a pre-DC. Splenic pre-DCs, which have lost pDC potential, generate only CD8\(^+\) cDCs when they are CD24\(^{\text{high}}\) and produce only CD8\(^-\) cDCs when they are CD24\(^{\text{low}}\) (Naik et al., 2006).
Figure 1.2 Development of conventional and plasmacytoid dendritic cells from mouse bone marrow progenitors. Although DCs were originally thought to originate from the myeloid lineage, recent evidence suggests that pDCs and cDCs can develop from myeloid and lymphoid precursors. In fact, DCs can be produced by precursors thought to be committed to a T cell restricted lineage. Adapted from references in section 1.7.
The most important cytokines in DC development are GM-CSF and Flt3L, although others have also been implicated (Lutz, 2004). In culture, GM-CSF administration to bone marrow cells or monocytes generates DCs and is often used as a prototype for the study of DC development and function (Caux et al., 1996; Sallusto and Lanzavecchia, 1994). As a result, it was very surprising when it was discovered that GM-CSF-deficient mice and mice deficient for the GM-CSF receptor have normal levels of DCs in lymphoid tissues (although GM-CSF receptor deficient mice have reduced LCs) (Vremec et al., 1997). This suggests that GM-CSF may not play a role in the steady-state development of DCs in vivo.

In contrast, the cytokine Flt3L (the ligand for Flt3, which, as discussed, is important for selection of cells with DC potential) has been shown to be critical for steady state development of both pDCs and cDCs. Flt3L-deficient mice have greatly reduced pDCs, CD8⁺ DCs and CD8⁻ cDCs (McKenna et al., 2000), while administration of Flt3L to mice increases all of these populations (Maraskovsky et al., 1996). Flt3L binds to the extracellular domain of Flt3, causing receptor dimerization and phosphorylation and activation of several downstream proteins (Gilliland and Griffin, 2002). Included in these proteins is the transcription factor, signal transducer and activator of transcription 3 (STAT3). Loss of STAT3 leads to a drastic reduction in DC numbers that cannot be overcome with administration of Flt3L (Laouar et al., 2003) and thus illustrates the importance of STAT3 as a checkpoint in Flt3L signalling as well as the overall significance of Flt3L in DC development.

Although initial studies indicated that M-CSF-deficient mice had little perturbation of DC numbers (but significant decreases in macrophages) (Wiktor-Jedrzejczak et al., 1990), more recent studies have found that M-CSF receptor (c-fms)-deficient mice lack both monocytes and LCs (Shortman and Naik, 2007). In addition, when mice are treated with M-CSF, pDC and cDC development can occur in a Flt3L-independent manner (Fancke et al., 2008). Mice that lack TGFβ are also deficient in LCs suggesting unique developmental features of this DC subtype (Shortman and Naik, 2007). It is also noteworthy that the M-CSF receptor is expressed by both pDCs and...
cDCs and isolated lin−ckit^{int}Flt3^{−}M-CSFR^{+} cells are capable of both in vitro and in vivo generation of pDCs and cDCs but no other lineages (Onai et al., 2007).

Several transcription factors have been implicated in DC development: Notable are members of the interferon regulatory factor family (IRF) (reviewed in (Gabriele and Ozato, 2007)). IRF8 deficient mice have significantly reduced numbers of pDCs (Tsujimura et al., 2003) and CD8^{+} DCs (Aliberti et al., 2003). Conversely, mice that are deficient for IRF2 or IRF4 have selective deficiencies in CD8^{−} (Suzuki et al., 2004) and CD4^{+} cDCs (Ichikawa et al., 2004). Related to the phenotype described for TGFβ-deficient mice, mice that are deficient for the helix-loop-helix transcription factor, inhibitor of DNA binding 2 (ID2), also lack LCs, since TGFβ induces ID2 expression, and have fewer CD8^{+} DCs (Hacker et al., 2003). Lastly, mice deficient in the transcriptional repressor Gfi1 have a significant reduction in all DC subsets (except for increased LCs), as well as DC-function impairments (Rathinam et al., 2005). In vitro, Gfi1-deficient hematopoietic progenitors are unable to differentiate into DCs and produce macrophages instead (Rathinam et al., 2005). The defect observed in these progenitors has been associated with a decrease in the activation of STAT3 (Rathinam et al., 2005). STAT3 is also downstream of Flt3 signalling and, as mentioned, a deficiency in this transcription factor also results in reduced pDC and cDC numbers (Laouar et al., 2003).

Taken together, these data illustrate that several different pathways are involved in the generation of the different DC subsets. As well, the multitude of progenitors that can generate DCs from both myeloid and lymphoid lineages points to the relative importance of these cells in the immune system.

**1.8 In vitro models of DC differentiation**

Since very few DCs can be isolated from tissues, several in vitro culture systems have been developed to study their development and function. The first system uses GM-CSF with or without other factors such as IL-4 (Caux et al., 1992; Inaba et al., 1992; Lutz et al., 1999; Sallusto and Lanzavecchia, 1994). Monocytes or BM cells can be cultured
with GM-CSF to generate a relatively homogenous population of CD11c<sup>+</sup>CD11b<sup>+</sup>CD8<sup>-</sup> MHCII<sup>+</sup> “myeloid” or cDCs (Naik, 2008). DCs derived in this manner have often been used as the canonical DC for studying development and function of in vivo CD8<sup>-</sup> cDCs. As already mentioned, however, mice deficient for GM-CSF or its receptor have a surprisingly normal DC phenotype suggesting that, in a steady state, GM-CSF may be largely dispensable for DC development in vivo (Hikino et al., 2000; Vremec et al., 1997). Peripheral plasma GM-CSF levels in steady state mice are below detectable limits. However, this does not necessarily mean that the levels are not locally sufficient for DC generation (Naik, 2008) and upon infection, levels of GM-CSF rise (Cheers et al., 1988). Thus, cells generated in the presence of GM-CSF may represent more of an inflammatory type DC.

Since some of the initial studies described above, it has become clear that Flt3L-derived DCs are a more accurate representation of steady state cDCs and pDCs. Bone marrow cells cultured in the presence of Flt3L are phenotypically and functionally distinct from those derived in GM-CSF (Xu et al., 2007). GM-CSF DCs are very large, granular and irregular shaped, while Flt3L-derived DCs are small and uniform (Xu et al., 2007). Flt3L cultures are found to contain three DC subtypes, CD11c<sup>+</sup>B220<sup>+</sup> pDCs, CD11c<sup>high</sup>CD11b<sup>low</sup> cDCs and CD11c<sup>low</sup>CD11b<sup>high</sup> cDCs, none of which express either CD4 or CD8 (Brasel et al., 2000; Brawand et al., 2002; Gilliet et al., 2002). Further marker analysis of the cDCs in the culture revealed that they could be further divided into CD24<sup>high</sup>Sirpα<sup>-</sup> and CD24<sup>low</sup>Sirpα<sup>+</sup>, which were subsequently determined to be excellent phenotypic and functional equivalents of CD8<sup>+</sup> and CD8<sup>-</sup> cDCs, respectively (Naik, 2008). Analysis of the DC precursors in Flt3L bone marrow cultures revealed two sequential DC-committed precursors that could give rise to all three DC subtypes (pDCs, CD8<sup>+</sup> equivalent and CD8<sup>-</sup> equivalent) (Naik et al., 2007). The first committed step was that of a proliferating pro-DC which differentiates into a transitional pre-DC and finally into all three DC subtypes (Naik et al., 2007).

In culture, GM-CSF is only capable of generating cDCs while Flt3L generates both pDCs and cDCs. The addition of GM-CSF to Flt3L cultures prevents pDC
development (Gilliet et al., 2002). Recent studies have elucidated the molecular mechanisms of GM-CSF induced pDC inhibition: GM-CSF activates STAT5, which directly inhibits IRF8 transcription (which, as mentioned above, is important for pDC and CD8\(^+\) DC development) and directly or indirectly inhibits the transcription factors Spi-B and IRF7, Flt3 receptor and the pathogen receptor, TLR9 (Esashi et al., 2008). In order to drive cDC development, GM-CSF also activates STAT3 and IRF4 expression. It has recently been shown that STAT3 is absolutely required for Flt3L-induced DC development while STAT5 is dispensable. In GM-CSF cultures, on the other hand, STAT3 is dispensable and STAT5 represses pDC development (Esashi et al., 2008). In the absence of STAT5, pDCs will develop in GM-CSF cultures (Esashi et al., 2008).

Lastly, M-CSF has recently been shown to be a novel factor for pDC and cDC development both *in vitro* and *in vivo* (Fancke et al., 2008). DCs with pDC features were found within the nonadherent cell fraction of M-CSF cultures. Moreover, BM precursors were capable of generating pDCs and cDCs *in vitro* when cultured with M-CSF even in Flt3L-deficient cells (Fancke et al., 2008).

### 1.9 Innate activation of DCs

Higher vertebrates are equipped with two defence systems designed to protect against pathogen infection. The first line of defence involves the evolutionarily ancient innate immune system, which functions by recognizing highly conserved structures specific to microbes, termed pathogen associated molecular patterns (PAMPs), by a set of germ-line encoded pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). This system unleashes a blunt force attack against microbial infection and is carried out primarily by macrophages, mast cells, neutrophils and NK cells. The second line of defence is the more recently evolved adaptive immune system, which is only present in vertebrates and employs B and T cells which express a diverse set of somatically rearranged receptors (BCRs and TCRs, respectively), allowing them to recognize a virtually limitless number of Ags (Janeway et al., 2005). Between these two systems are DCs whose Ag-presenting functions effectively link innate recognition of pathogens to
the generation of appropriate adaptive immune responses by B and T cells (Lee and Iwasaki, 2007).

In an immature state, DCs are found throughout the body, and, in particular, at portals of pathogen entry such as the spleen (which filters the blood), the skin and mucosal surfaces (Sabatte et al., 2007). Upon encounter with a microbial invader, a PAMP will activate one of the PRRs present on the DC. There are several classes of PRR expressed by DCs, including C-type lectin receptors (CLRs), scavenger receptors (SRs) and Toll-like receptors (TLRs) (which will be the focus of this section). CLRs are responsible for the recognition of carbohydrate structures on pathogens and their internalization for processing and presentation by DCs. Included in the CLRs expressed by DCs are the mannose receptor (MR) (Sallusto et al., 1995), CD205 (Mahnke et al., 2000), DC-specific ICAM3-grabbing non-integrin (DC-SIGN) (Geijtenbeek et al., 2000), BCDA-2 (Dzionek et al., 2001), DC-associated molecule-1 (DECTIN-1) (Ariizumi et al., 2000), DC immunoreceptor (DCIR) (Bates et al., 1999), DC-associated lectin-1 (DCAL-1) (Ryan et al., 2002) and C-LEC (Colonna et al., 2000), all of which vary in expression, depending on the DC subtype. SRs are a diverse set of receptors which bind to polyanionic ligands and function to clear lipoproteins and take up pathogens. DCs express class-A scavenger receptors (Harshyne et al., 2003), CD36 (class B scavenger receptors) (Albert et al., 1998) and LOX-1 (Delneste et al., 2002).

1.9.1 Discovery of Toll-like receptors

TLRs owe their name to the Drosophila gene, Toll, which was identified in the early 1980s in a genetic screen to find the pathway involved in patterning dorso-ventral polarity in the fruit fly embryo (Galiana-Arnoux and Imler, 2006). Loss-of-function and gain-of-function mutations of Toll resulted in dorsalizing and ventralizing phenotypes, respectively, and was thus named from the German word ‘terrific’, since this gene was of critical importance to the pathway.
Toll is a type I transmembrane receptor with an ectodomain composed of leucine-rich repeats (LRRs) capped on each end by characteristic N- and C-terminal motifs (Galiana-Arnoux and Imler, 2006). The cytoplasmic domain of Toll shares high sequence similarity to the Type I receptor for interleukin (IL)-1 and is thus called the TIR (Toll/IL-1R) domain (Gay and Keith, 1991). It wasn’t until many years later that Toll was shown to play a role in the immune response of Drosophila. Predating this finding were studies showing that several potent antimicrobial peptides were secreted in response to septic injury and that expression of the genes encoding these peptides was regulated by nuclear factor kappa B (NFκB) (Georgel et al., 1993; Reichhart et al., 1993). It was subsequently found that Toll mutant flies failed to express the anti-microbial peptide Drosomycin in response to infection and were highly susceptible to fungal infections (Lemaitre et al., 1996). From this point, the search for a mammalian homologue was on.

In 1997, the first human Toll homologue was discovered (Medzhitov et al., 1997). It was shown to be capable of activating NFκB and triggering the synthesis of inflammatory cytokines and co-stimulatory molecules (Medzhitov et al., 1997). The discovery of this receptor, now known as Toll-like receptor (TLR) 4, paved the way for Toll receptors to be the prime candidates to fulfill the function of PRRs, ie., germline encoded proteins evolutionarily selected to recognise PAMPs, as was proposed by Charles Janeway years earlier (Janeway, 1989).

Genetic proof that TLR4 was in fact a critical innate immune receptor came from studies to determine the genetic basis of the resistance to LPS-induced sepsis in C3H/HeJ mice (Heppner and Weiss, 1965). Positional cloning analysis revealed a point mutation in the tlr4 gene in which a conserved proline in the TIR domain was changed to a histidine, preventing downstream signalling (Poltorak et al., 1998). The TLR family now consists of 10 members in humans and 12 in mice and the generation of knockout mice for the various TLRs has enormously facilitated the characterization of their functional properties.
1.9.2 TLR1, TLR2 and TLR6 function

TLR2 recognizes a variety of microbial components. These include lipoproteins and lipopeptides from various bacteria, peptidoglycan (PGN) from Gram-positive bacteria, lipoarabinomannan from *Mycobacterium tuberculosis*, GPI anchors from *Trypanosoma cruzi*, a phenol soluble modulin from *Staphylococcus epidermis*, porins from *Neisseria meningitides*, glycolipids from *Treponema maltophilum* and the yeast cell wall component zymosan (Akira et al., 2001; Medzhitov, 2001; Takeda et al., 2003). In addition, reports suggest that TLR2 can also recognize LPS from non-enterobacteria (Werts et al., 2001) which differ structurally from LPS from Gram-negative bacteria (recognized by TLR4) by the number of acyl chains in the lipid A component (Netea et al., 2002).

TLR2 is capable of recognizing such a diverse set of microbial components, in part, because of its association with TLR1 and TLR6, both of which are structurally related to TLR2. MΦs derived from TLR6-deficient mice do not respond to mycoplasma-derived diacyl lipopeptides (mycoplasmal MΦ-activating lipopeptide-2kD (MALP-2)), but show normal responses to triacyl lipopeptides (Takeuchi et al., 2001). On the other hand, MΦs derived from TLR1-deficient mice have normal responses to mycoplasma-derived diacyl lipoproteins, but an impaired response to triacylated lipopeptides (Takeuchi et al., 2002). Thus, TLR2 can form heterodimers with both TLR1 and TLR6 in order to discriminate and differentially respond to triacylated and diacylated lipoproteins, respectively.

1.9.3 TLR3 function

Within a cell, viral replication often results in the generation of double stranded (ds) RNA and this triggers the synthesis of type I interferons (IFNα/β) which exert antiviral and immunostimulatory effects. TLR3-deficient mice have reduced responses to dsRNA and to the synthetic analogue poly (I): poly(C) (Alexopoulou et al., 2001). Thus,
TLR3 is implicated in the recognition of dsRNA and viruses. Other TLR3-independent mechanisms of dsRNA recognition exist however and will be discussed later.

1.9.4 TLR4 function

As mentioned above, TLR4 is an essential receptor for the recognition of LPS from Gram-negative bacteria. TLR4, however, is not sufficient for LPS-induced signalling. MD-2, which associates with the extracellular domain of TLR4, is also necessary (Nagai et al., 2002). In general, for LPS-induced signalling to take place, LPS is first captured by an LPS-binding protein (LBP) present in plasma (Schumann et al., 1990) and taken to CD14 on the surface of the responding cell (Wright et al., 1990). Although LPS/LBP bound to CD14 is incapable of transmitting signals (CD14 is a GPI-anchored protein and thus lacks an intracellular domain) it delivers the LPS to the TLR4/MD-2 complex which then initiates the intracellular signalling cascades required for LPS responsiveness.

1.9.5 TLR5 function

TLR5 recognizes an evolutionary conserved domain within flagellin, the 55kDa monomeric form of bacterial flagella from both Gram-positive and Gram-negative bacteria (Hayashi et al., 2001). TLR5 is expressed on the basolateral, but not the apical, surface of intestinal epithelia (Gewirtz et al., 2001), on intestinal endothelial cells of the sub-epithelial compartment (Maaser et al., 2004) and on lung epithelial cells (Hawn et al., 2003). Thus, TLR5 has an important role in bacterial recognition at mucosal surfaces.

1.9.6 TLR7 and TLR8 function

TLR7 and TLR8 are structurally related proteins which recognize many of the same ligands. TLR8 is thought to be non-functional in mice (Heil et al., 2004). TLR7 in mice (and both TLR7 and TLR8 in humans (Jurk et al., 2002)) was initially found to be activated by imidazoquinolines, which are guanosine-related drugs used primarily for the
treatment of virally-induced genital warts (Hemmi et al., 2002). Murine TLR7 has also been shown to recognize the synthetic compound loxoribine, which has anti-tumour effects (Heil et al., 2003). These receptors are required for the induction of type I IFNs in response to RNA viruses, but, unlike TLR3 which responds to dsRNA, TLR7 and TLR8 are activated by single stranded (ss) RNA rich in guanosine or uridine residues (Heil et al., 2004). Although ssRNA is also present in uninfected host cells, it does not illicit a response via TLR7 or TLR8 likely because these receptors are only present in endosomes and host-derived ssRNA is not normally found in this compartment (Takeda and Akira, 2005).

1.9.7 TLR9 function

Analysis of TLR9-deficient mice determined that TLR9 is a receptor for bacterial (and viral) DNA, as well as synthetic oligodeoxynucleotides containing non-methylated CpG-dinucleotides (CpG DNA) (Hemmi et al., 2000). Bacterial DNA contains many CpG motifs, whereas the frequency of CpG motifs is considerably reduced in vertebrates and the cysteine residues within them are highly methylated (Takeda and Akira, 2005). Thus, TLR9 is able to distinguish bacterial CpG and respond to it. Interestingly, studies with synthetic CpG DNA have shown that those composed entirely of phosphorothioate linkages (B/K-type CpG DNA) potently induce IL-12 and TNFα (Hemmi et al., 2003; Verthelyi et al., 2001) while those containing phosphorothioate G-rich sequences at the ends and phosphodiester palindromic sequences with a CpG dinucleotide in the middle (A/D-type CpG DNA) are more potent at inducing IFNα production from pDCs (Hemmi et al., 2003; Krug et al., 2001; Verthelyi et al., 2001). TLR9 is essential for the recognition of both types of these CpG DNAs as indicated by the unresponsiveness of TLR9 knockout mice to both forms (Hemmi et al., 2003).

1.9.8 TLR11 function

TLR11 is present in mice but not humans and is known to recognize uropathogenic *Escherichia coli* (Lauw et al., 2005). The first defined ligand for TLR11
was a profilin-like protein from the protozoan parasite *Toxoplasma gondii* (Yarovinsky et al., 2005). Activation of TLR11 by this profilin-like molecule in DCs results in the generation of IL-12. As well, TLR11 is required for *in vivo* parasite induced IL-12 production and for optimal resistance to protozoan infection (Yarovinsky et al., 2005).

1.9.9 TLR signalling

TLR activation results in a potent immunostimulatory response. TLRs occur as dimers (Ozinsky et al., 2000) and are thought to be pre-assembled in a low-affinity complex before ligand binding. Since TLR ligands are monomeric, it is thought that the dimerization of the ectodomains is symmetrical and this causes the cytosolic TIR domains to also associate symmetrically (Ozinsky et al., 2000). Upon ligand binding, a conformational change brings the TIR domains of each receptor into closer proximity and the TIR domains undergo structural reorganization, creating the stage necessary for adapter recruitment and subsequent signalling leading to transcriptional activation (Ozinsky et al., 2000). The TIR domain containing adapters involved in TLR signalling include myeloid differentiation primary-response gene 88 (MyD88), MyD88-adapter-like (MAL, also known as TIRAP), TIR-domain-containing adapter protein inducing IFNβ (TRIF, also known as TICAM1), TRIF-related adapter molecule (TRAM, also known as TICAM2) and sterile α-and armadillo-motif-containing protein (SARM) (O’Neill and Bowie, 2007). SARM is an inhibitor of TLR signalling, interacting with TRIF and interfering with its function (Carty et al., 2006). MyD88 is shared by all TLRs except TLR3 (evident by MyD88-deficient mice being largely unresponsive to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9). MAL/TIRAP only binds to TLR2 and TLR4, TRIF/TICAM1 binds only to TLR3 and TLR4 while TRAM/TICAM2 binds exclusively to TLR4 (O’Neill and Bowie, 2007) (Fig 1.3). In general, TLR signalling can be divided into MyD88-dependent, which culminates in the activation of NFκB, or MyD88-independent, which results in activation of interferon regulatory factors (IRFs) and production of Type I IFNs.
MyD88-dependent signalling

In response to ligands that bind TLR2 (Takeuchi et al., 2000), TLR4 (Kawai et al., 1999; Takeuchi et al., 2000), TLR5 (Hayashi et al., 2001), TLR7 (Hemmi et al., 2002) or TLR9 (Schnare et al., 2000) MyD88 is recruited via its TIR domain (Medzhitov et al., 1998) (either directly or through the bridging adaptor MAL/TIRAP (Yamamoto et al., 2002a)). Immediately downstream of MyD88 is IL-1R-associated kinase (IRAK) 4 which recruits IRAK1 (Janssens and Beyaert, 2003; Wesche et al., 1997). IRAK4 phosphorylates IRAK1 and the kinases dissociate from MyD88. Tumour necrosis-factor-
receptor associated factor 6 (TRAF6) is the downstream target for IRAK1. Binding of IRAK1 to TRAF6 is a key step in the activation and recruitment of transforming-growth-factor-β-activated kinase 1 (TAK1) and TAK1-binding protein 2 (TAB2) (Takaesu et al., 2000) and the ubiquitinylating factors ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13) (O’Neill and Bowie, 2007). TAK1 activates the inhibitor of NFκB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ (Wang et al., 2001). The activated IKK complex phosphorylates inhibitor of κB (IκB) leading to its ubiquitination and degradation. This releases NFκB to translocate into the nucleus and induce expression of inflammatory cytokines. TAK1 also has the ability to act as a mitogen activated protein kinase (MAPK) kinase and activate MAPK kinases 3/6 and 4/7, which, in turn, activate p38MAPK and stress activated protein kinase (SAPK)/ JUN N-terminal kinase (JNK) (Wang et al., 2001). In addition, by an unknown mechanism, another MAPK kinase kinase, Cot/Tpl2, phosphorylates and activates MEK1/2 in response to TLR stimulation and leads to the activation of extracellular signal-related kinase 1/2 (Erk1/2) (Sugimoto et al., 2004).

It has also been shown that another member of the IRAK family, IRAK2, is also important in TLR signalling downstream of IRAK4 (Kawagoe et al., 2008). Although IRAK2 is dispensable for the initiation of TLR-induced signalling cascades, it is essential for sustaining TLR-induced activation of NFκB and cytokine production (Kawagoe et al., 2008). Unlike IRAK1 whose activation peaks at 1 hour after TLR stimulation, IRAK2 kinase activity is sustained and peaks at 8 hours after stimulation (Kawagoe et al., 2008). It appears that IRAK1 and IRAK2 act in sequence with both being essential for TLR2-induced cytokine production (Kawagoe et al., 2008). However, TLR3 signalling appears to be normal in IRAK2-deficient mice, and no role for IRAK2 in TLR3 signalling has been found (Kawagoe et al., 2008).

MyD88-dependent signalling pathways are essential for the activation of IRF7 and production of Type I IFNs downstream of TLR7, TLR8 and TLR9 (Kawai et al., 2004). A complex consisting of MyD88, IRAK1, IRAK4, TRAF6 and IRF7 has been detected in which IRAK1 phosphorylates IRF7, leading to its activation and translocation
to the nucleus (Kawai et al., 2004). MyD88 also interacts with IRF5 (Takaoka et al., 2005) and IRF1 (Negishi et al., 2006) and is required for their activation. IRF5 interacts with and is activated by MyD88 and TRAF6 and results in the induction of proinflammatory cytokines such as IL-6, IL-12 and TNFα (Takaoka et al., 2005).

**MyD88-independent signalling**

TRIF is a key adapter used in MyD88-independent TLR signalling (Hoebe et al., 2003a; Yamamoto et al., 2002b). It is the exclusive adapter used by TLR3 and is also used by TLR4 (in conjunction with TRAM (Yamamoto et al., 2003b)) (Oshiumi et al., 2003; Yamamoto et al., 2002b). Stimulation of TLR3 on endosomal membranes and TLR4 on the cell surface activates pathways that result in the activation of IRF3 and production of Type I IFNs and subsequent expression of IFN-inducible gene products (Oshiumi et al., 2003; Yamamoto et al., 2002b; Yamamoto et al., 2003a). Upon activation of TLR3 or TLR4, TRIF associates with TRAF-family member associated NFκB activator binding kinase 1 (TBK1) through NAK-associated protein 1 (NAP1) (Sasai et al., 2005) and possibly TRAF3. The nuclear translocation of IRF3 is triggered by phosphorylation by TBK1 and IKKι/ε (Akira et al., 2006). Upon phosphorylation, IRF3 dimerizes and translocates to the nucleus (Akira et al., 2006) (Fig 1.3).

NFκB activation downstream of TRIF can occur via two different mechanisms. One route is through a TRAF6-binding motif in the N-terminal region of TRIF which, when mutated, decreases TRIF-induced NFκB, but not IRF3, activation (Jiang et al., 2004; Sato et al., 2003b). However, the role of TRAF6 in TRIF-dependent signalling is still controversial. The second route to NFκB activation involves the C-terminus of TRIF which contains a receptor-interacting protein (RIP) homotypic interaction motif (RHIM). TRIF recruits RIP1, which is required for induction of NFκB activation (Meylan et al., 2004).

TLR4 activates both the MyD88-dependent pathway through MAL-MyD88 and the MyD88-independent pathway through TRIF-TRAM. This results in the production of
pro-inflammatory cytokines and type I IFNs, respectively. Recent studies have revealed that cell compartment localization of TLR4 determines which pathway becomes activated (Kagan et al., 2008; Tanimura et al., 2008). TLR4 at the plasma membrane engages first with MAL/TIRAP followed by MyD88 for NFκB activation. TLR4 is then endocytosed where TRAM is engaged allowing the recruitment of TRIF (Kagan et al., 2008). This internalized signalling complex co-localizes with TRAF3 to activate TBK1 from the early endosome (Tanimura et al., 2008). Binding of MAL and TRAM to TLR4 appear to be mutually exclusive events (Nunez Miguel et al., 2007) and this is consistent with reports showing that TLRs that activate TBK1 are cytosolic or endosomal and suggests that all signalling for Type I IFN production originates from intracellularly located TLRs.

**Negative regulation of TLR signalling**

TLR activation is a very potent mechanism of innate immune activation and generates high levels of pro-inflammatory cytokines. As a result, careful regulation is required to prevent sustained TLR-induced signalling from cascading out of control to cause septic shock or autoimmune disorders (Lang and Mansell, 2007).

Several mechanisms exist to negatively regulate TLR signalling. The only extracellular mechanism involves soluble decoy TLRs (sTLRs) which are the first line of negative regulation for TLR signalling (Colotta et al., 1994). sTLRs can compete for TLR agonists, or, in the case of TLR4, can block the interaction of TLR4 with MD2 and CD14, preventing downstream signalling (Hyakushima et al., 2004). Additionally, transmembrane protein regulators (eg. suppressor of tumorigenicity 2 (ST2)), which either sequester adaptors required for TLR signalling or interfere in ligand binding, can also negatively regulate TLR signalling (Lang and Mansell, 2007).

Several intracellular negative regulators of TLR signals have been identified. Included in these is MyD88 short (MyD88s), a splice variant of the adapter MyD88 (Janssens et al., 2002; Janssens et al., 2003). Overexpression of MyD88s results in inhibition of IRAK4 and subsequent phosphorylation of IRAK1 (Burns et al., 2003).
SARM is also implicated as a negative regulator of NFκB activation downstream of TRIF, and LPS treatment results in increased levels of SARM to attenuate LPS-induced events (Carty et al., 2006). A kinase dead IRAK family member, IRAK-M (Janssens et al., 2003), prevents dissociation of IRAK1/IRAK4 from the MyD88 receptor complex and thus blocks the association of IRAK1 with TRAF6 and thus further downstream signalling (Kobayashi et al., 2002). Toll-interacting protein 1 (Tollip1) binds to TLR2 and TLR4 and interacts with IRAK1 to inhibit NFκB activation (Zhang and Ghosh, 2002). Suppressor of cytokine signalling-1 (SOCS-1) also negatively regulates TLR2 and TLR4 by targeting MAL/TIRAP for polyubiquitination and degradation (Mansell et al., 2006). Lastly, SH2 containing protein tyrosine phosphatase-2 (SHP-2) has been implicated in the negative regulation of IFNβ production by TLR3 and TLR4 (An et al., 2006).

1.9.10 DCs and TLRs

TLR expression varies with DC subset. In mice, all splenic DC subsets express TLR1, TLR2, TLR4, TLR6, TLR8 and TLR9 (Table 1.1) (Hochrein and O'Keeffe, 2008). Splenic pDCs, however, express either low or no TLR3, but have high expression and responsiveness to TLR7 and TLR9, which are paramount to their function (Edwards et al., 2003; Naik et al., 2005). Meanwhile, CD8+ DCs lack or have low TLR5 levels, do not express TLR7 and fail to respond to imidazoquinolines (Edwards et al., 2003). In addition, there are distinct differences in the expression of TLRs between freshly isolated DCs and those derived in culture. For example, splenic DCs express low levels of TLR4 and are largely unresponsive to LPS, whereas GM-CSF-derived DCs express high levels of TLR4 and generate robust responses to LPS stimulation (Boonstra et al., 2003).

For the most part, analysis of TLR expression in DCs has relied upon RT-PCR since there are few reliable anti-TLR antibodies. However, expression at the mRNA level does not necessarily translate to protein expression and responsiveness to a given TLR ligand. In addition, there are discrepancies in the literature concerning TLR expression in DCs, likely due to different methods used to purify cells or possible impurities in the
subsets obtained. Also, not all reports have looked for expression of every TLR in every DC subset. In particular, studies examining TLR expression in \textit{in vitro} BM-derived DCs are sparse.

In terms of TLR signalling, much of the work to delineate the pathways involved has been done with MΦs and recent studies have revealed important differences between MΦs and DCs. For example, a recent study using both \textit{in vitro} and \textit{in vivo} methods and two forms of TRIF mutants, demonstrated, in agreement with previous reports (Hoebe et al., 2003b), that upregulation of co-stimulatory molecules is abrogated in MΦs when TRIF is absent (Shen et al., 2008). However, DCs are still capable of upregulating co-stimulatory molecules in the absence of TRIF but maximal T cell priming by LPS-stimulated DCs requires both MyD88 and TRIF pathways (Shen et al., 2008). Thus, in DCs, MyD88 and TRIF pathways act synergistically to maximize DC maturation and induce T cell activation (Shen et al., 2008).

The differences found to date in TLR expression and pathway activation within DC subsets suggest differences in pathogen recognition and perhaps unique roles in shaping adaptive immune responses.

\textbf{1.9.11 TLR-independent responses}

Receptors other than TLRs are also capable of initiating innate immune responses and sensing infection. This is valuable because there are no “free” TLRs in the cytosol and so it is important to have a second means of pathogen detection for those microbes that have penetrated into the cytosol, as is often the case for viruses. The first cytosolic sensor for viral RNA discovered was protein kinase R (PKR) (Hovanessian, 2007). PKR phosphorylates eIF2\(\alpha\), preventing translation of viral and host cellular proteins. PKR activity does not, however, explain TLR-independent NF\(\kappa\)B activation. This was subsequently discovered to be due to retinoic acid inducible gene 1 (RIG1), an RNA helicase that regulates Type I IFNs in response to dsRNA (Yoneyama et al., 2004)(Kato et al., 2005). A second RNA helicase, melanoma differentiation associated gene 5
(mda5), was also shown to be involved in sensing dsRNA in the cytosol (Yoneyama et al., 2005). RIG1 employs the same signalling pathways to induce Type I IFNs as TLRs, ie., through TRAF6 and the TBK1/IKKε pathway (Kato et al., 2005). The transcription factors activated by this pathway, ie., NFκB/AP1 and IRF3 initiate the immune response (Kato et al., 2005). RIG1 has been found to be a critical regulator of Type I IFN production in several cell types, except pDCs, where TLRs are primarily used to sense RNA viruses (Kato et al., 2005). Finally, the cytosolic receptors Nod1 and Nod2, that belong to the Nod like receptor (NLR) family, have been shown to recognize cytosolic peptidoglycan (Kufer et al., 2005). Nod1 and Nod2 regulate activation of NFκB through the kinase RIP2, which phosphorylates the IKK complex leading to NFκB activation (Kufer et al., 2005).

1.10 Linking innate activation of DCs to adaptive immune responses

Stimulation of DCs by a pathogen, through any of the mechanisms described above, results in DC maturation (Reis e Sousa, 2006). This maturation process is complex and enables DCs to trigger the activation of naïve T cells. The nature of the maturing stimulus promotes DC maturation into different functional profiles.

The maturation of DCs is associated with several coordinated events. In response to PRR activation, DCs express the lymph node homing chemokine receptor CCR7 which facilitates their migration through lymphatic vessels to the nodes (Ohl et al., 2004). CCR7 is the receptor for the chemokines CCL19 and CCL21, which are highly expressed in T cell rich areas of the lymph nodes (Randolph et al., 2005; Sanchez-Sanchez et al., 2006). This process is aided by inflammatory mediators such as prostaglandin E2 (PGE₂) and the ADP-ribosyl cyclase, CD38. PGE₂ and CD38 sensitize CCR7 to CCL19 and CCL21 (Randolph et al., 2005; Sanchez-Sanchez et al., 2006).

Maturing DCs decrease their ability to capture and process Ags. This feature ensures that T cells will be presented with Ags that were acquired in the context of PRR stimulation in the periphery (Lee and Iwasaki, 2007; Reis e Sousa, 2006; Sabatte et al.,
In addition, the maturational process results in increased cell surface expression of MHC-peptide complexes, CD40, the co-stimulatory molecules CD80/CD86 and the integrin lymphocyte function-associated antigen 1 (LFA-1) (Lee and Iwasaki, 2007; Reis e Sousa, 2006; Sabatte et al., 2007). Lastly, maturing DCs are stimulated to produce and secrete high levels of pro-inflammatory cytokines (such as TNFα, IL-12 and IL-6) and chemokines (Lee and Iwasaki, 2007; Reis e Sousa, 2006; Sabatte et al., 2007).

Mature DCs deliver three signals to naïve T cells that direct their response. Signal 1 is delivered by the MHC-peptide complex on the surface of the DC to the TCR (Lee and Iwasaki, 2007; Reis e Sousa, 2006; Sabatte et al., 2007). Signal 2 is via the co-stimulatory molecules CD80/CD86 expressed on the DC surface to CD28 on the T cell. Signal 3 is delivered by cytokines secreted by DCs in response to pathogen recognition that helps direct the differentiation of T cells into Th1, Th2, Th17 or cytotoxic T effector cells (Lee and Iwasaki, 2007; Reis e Sousa, 2006; Sabatte et al., 2007).

Evidence for the requirement of all three signals has come from studies using MyD88-deficient DCs (Kaisho et al., 2001). In these studies, DCs derived from MyD88-deficient mice were stimulated with extract from Mycobacterium. Myd88-/- DCs failed to mature in vitro and, in vivo, fail to activate T cells and induce IFNγ production. To illustrate the importance of cytokine production, MyD88-deficient DCs were stimulated with LPS (which activates both MyD88-dependent and independent pathways). This treatment led to DC maturation (by expression of mature surface markers) but not cytokine production. If MHC and co-stimulation were sufficient to activate naïve T cells, then these LPS-stimulated MyD88-deficient DCs should still have been capable of activating naïve T cells. This, however, was not the case as effective T cell priming was not achieved (Kaisho et al., 2001). Further studies revealed that the cytokine requirement was not simply to aid activation of T cells but to prevent suppression (Pasare and Medzhitov, 2003). Related to this, T cell responses are suppressed by CD4+CD25+ regulatory T cells (Tregs) which play a role in the maintenance of peripheral tolerance by inhibiting peripheral autoreactive T cells. Activation of DCs by LPS and CpG (acting through TLR4 and TLR9, respectively) blocks the suppressive effects of Tregs and
allows pathogen-specific adaptive immune responses (Pasare and Medzhitov, 2003). DC production of IL-6 in response to TLR activation is responsible, at least in part, for making responder T cells refractory to suppression (Pasare and Medzhitov, 2003).

In addition, DC cytokine production profiles induced by specific microbial stimuli affect their ability to induce either a T_{H1} or a T_{H2} response. T_{H1} cells are crucial for cellular immunity against intracellular pathogens while T_{H2} cells are essential for humoral immunity and defence against helminth and parasitic infections (Sabatte et al., 2007). DC-secreted IL-12, IL-18 and IFN\(\alpha\) induce T_{H1} responses characterized by the production of high levels of IFN\(\gamma\) that acts to suppress the generation of T_{H2} responses. T_{H2} responses, on the other hand, are characterized by IL-4, IL-5 and IL-13 production and these cytokines act to inhibit differentiation of T_{H1} cells (Lee and Iwasaki, 2007; Reis e Sousa, 2006; Sabatte et al., 2007). Using human DCs, differential activation of the MAPK pathway by TLRs has been shown to direct T_{H} responses. Specifically, activation of p38MAPK and c-Jun by LPS and flagellin (via TLR4 and TLR5, respectively) induces the production of IL-12p70 and stimulation of a T_{H1} response (Agrawal et al., 2003). By contrast, sustained activation of Erk1/2 results in the stabilization of c-Fos in response to the TLR2 agonist, Pam\(_3\)Cys, and stimulates T_{H2} responses by suppressing IL-12 production (Agrawal et al., 2003; Dillon et al., 2004).

In addition to T_{H1} and T_{H2} cell polarization, DCs also play a role in the induction of the newly characterized T_{H17} T cell subtype. T_{H17} cells have a role in host defense against certain pathogens including Klebsiella, Citrobacter, Borrelia and fungi (Korn et al., 2007a). Mouse T_{H17} cells differentiate from naïve T cells in the presence of TGF\(\beta\), IL-1 and IL-6 and require IL-23 for survival and proliferation (Korn et al., 2007a). IL-23 is a member of the IL-12 cytokine family, and shares the p40 subunit with IL-12 which associates with its own specific p19 subunit (Kastelein et al., 2007). Both IL-12 and IL-23 are produced by activated DCs, but likely in response to different stimuli. Activation of DCs with PGE\(_2\), ATP or anti-CD40 antibody leads to enhanced IL-23 production and T_{H17} skewing (Schnurr et al., 2005; Sheibanie et al., 2004). In vitro differentiation studies have found that BM-derived DCs generated in the presence of PGE\(_2\) produce
reduced amounts of IL-12 and high IL-23 in response to LPS (Khayrullina et al., 2008). These conditions promote T<sub>H</sub>17 differentiation and inhibit T<sub>H</sub>1 and T<sub>H</sub>2 skewing, both in vitro and in vivo.

The majority of the studies described above were performed using cDCs. However, pDCs also have the ability to induce adaptive immune responses (Fitzgerald-Bocarsly et al., 2008). Activation of pDCs by TLR7 and TLR9 ligands also results in maturation, ie., increased expression of MHC molecules and co-stimulatory molecules. Upon maturation, pDCs also decrease their ability to produce IFN<sub>α</sub> and become more proficient at Ag presentation. In response to viral activation, pDCs produce high levels of IFN<sub>α</sub> and the pro-inflammatory cytokines IL-12, IL-6 and TNF<sub>α</sub>, and these conditions induce T<sub>H</sub>1 differentiation (Kadowaki et al., 2000).

DCs are capable of activating B cells and inducing humoral immunity although this ability is rarely studied. Specifically, cDCs are capable of promoting the differentiation of memory B cells into IgG and IgA secreting plasma cells (Dubois et al., 1997). IL-12 (Dubois et al., 1998) and IL-6 production by DCs are critical steps in this process. pDCs are also capable of promoting B cell differentiation (Jego et al., 2003). For example, pDCs stimulated with influenza virus produce type I IFNs and IL-6 and this, in turn, induces B cell differentiation into plasma cells (Jego et al., 2003).

DCs are invaluable as a link between microbial recognition and initiation of adaptive immune responses. In large part, activation of DCs by PRRs gains information about the nature of the infection allowing the DC to direct an appropriate T cell response. In addition, microbes often express several different PAMPs acting through different TLRs, thus it is not surprising that some TLRs synergize to enhance DC activation and ensure activation of an adaptive immune response (Trinchieri and Sher, 2007).
1.11 Dendritic cells in disease

Dysregulation of DC development and functions can lead to the manifestation of different types of diseases. In particular, intrinsic dysregulation can lead to autoimmune diseases and allergy (Th1 and Th2 diseases, respectively) (Ueno et al., 2007). Autoimmune diseases are chronic inflammatory conditions that result from inappropriate responses to self-Ags. One example of a human autoimmune disease is multiple sclerosis (MS), which is modelled in mice by experimental autoimmune encephalomyelitis (EAE) (El Behi et al., 2005). EAE pathology is characterized by T cells that are capable of recognizing myelin proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP) (El Behi et al., 2005). These T cells are activated in the periphery and migrate to the CNS where they cause autoimmune inflammation leading to ascending paralysis starting from the base of the spine. EAE has historically been classified as a Th1 MHCII-restricted CD4+ T cell-mediated disease of the CNS (El Behi et al., 2005). Recently, however, it has become clear that Th17 cells are critical for driving pathogenesis of this disease (Korn et al., 2007a; Langrish et al., 2005). Activated T cells lead to an alteration in the blood brain barrier (BBB) and allow the recruitment of other cell types such as monocytes, B cells and CD8+ T cells into the CNS (El Behi et al., 2005). In addition, cytokines produced by T cells activate MΦs and resident microglia, which results in increased destruction and demyelination of the CNS. Even though T cells play pivotal roles in EAE and MS, it is likely that other immune cells also propagate the pathology, in particular those cells involved in T cell priming.

It has been hypothesized that T cell-mediated attack against myelin is the result of inappropriate Ag presentation of either self-peptides or Ags capable of mimicking self-peptides in peripheral lymphoid organs (El Behi et al., 2005). Evidence for the involvement of DCs in EAE induction comes from experiments showing that myelin Ag-loaded DCs matured in vitro efficiently activate T cells upon adoptive transfer and result in induction of EAE (Weir et al., 2002). As well, both BM-derived DCs and isolated CD8+ DCs loaded in vitro with encephalitogenic peptides are capable of inducing paralysis upon transfer into naïve mice (Dittel et al., 1999). However, DCs, under some
circumstances, have also been shown capable of preventing clinical symptoms of EAE. For example, pretreatment of DCs with IFNγ prior to injection into recipient rats results in significant inhibition of clinical symptoms of EAE (Xiao et al., 2004). TGFβ-treated DCs also induce apoptosis of CD4+ T cells through the production of nitric oxide (NO) after in vitro co-culture (Jin et al., 2000). As well, DC subsets, such as CD8+CD4+, have been found to suppress EAE by the production of IL-10 that inhibits T cell function (Legge et al., 2002).

Thus DCs present a promising target for autoimmune immunotherapy. Manipulation and control of DC plasticity could be used to modify and maximize their tolerogenic properties to decrease disease severity or block an autoimmune flare.

1.11 Relationship to human DCs

In contrast to the plethora of mouse DC studies, there have been few studies carried out with freshly isolated human DCs due to the difficulty in acquiring them. Blood is the only readily accessible source both for direct isolation and derivation (Shortman and Liu, 2002). Therefore, because of these limitations, it is currently difficult to comprehensively compare mouse and human DC subtypes. Adding to the difficulty is the fact that human DCs do not express CD8, therefore, the human equivalent of mouse CD8+ DCs remains elusive. In the few cases where human lymphoid tissue DCs have been examined, the heterogeneity of CD11c, CD11b and CD4 marker expression appears to be similar to mouse. The blood DC Ag (BDCA) markers have facilitated increased distinction of human DC subsets (Dzionek et al., 2000) and will likely lead to further correlations between mouse and human DC subsets.

1.12 The Src homology 2 domain containing inositol 5′-phosphatase (SHIP)

The Src homology 2 containing inositol 5′-phosphatase, SHIP (also known as SHIP1), was first identified as a 145 kDa protein that becomes tyrosine phosphorylated and associated with Shc upon growth factor or cytokine receptor activation (Damen et al.,
The gene encoding SHIP is officially designated *Inpp5d*, but I have used the simplified name *Ship* in this thesis when referring specifically to the genomic locus. Structurally, SHIP possesses an amino-terminal Src homology 2 (SH2) domain that preferentially binds to phosphotyrosyl motifs containing the relatively broad consensus pY(Y/S/T/v)(L/y/nle/f)(L/Nle/L/V) (Sweeney et al., 2005; Wavreille et al., 2007). Known SH2-mediated binding partners include tyrosine phosphorylated Shc, SHP2, downstream of tyrosine kinases (Doks), Gabs, CD150, PECAM, Cas, c-Cbl (Pesesse et al., 2006), certain immunoreceptor tyrosine based inhibitory motifs (ITIMs), and certain immunoreceptor tyrosine based activation motifs (ITAMs) (Sly et al., 2003). SHIP also contains a centrally located phosphoinositide phosphatase domain that selectively hydrolyzes the 5′-phosphate of phosphatidylinositol (PI)-3,4,5-trisphosphate (PIP₃) and inositol-1,3,4,5-tetrakisphosphate (IP₄) (Pesesse et al., 2006). At its C-terminal end, SHIP possesses a critical proline-rich region that binds a subset of SH3-containing proteins including Grb2, Src, Abl, PLCγ1 and PIAS1 as well as two NPXY sequences that, when phosphorylated, bind phosphotyrosine binding (PTB) domains in proteins such as Shc, Dok 1 and 2, p85α and SHIP2 (Rohrschneider et al., 2000; Sly et al., 2003; Song et al., 2005; Wisniewski et al., 1999) (Fig 1.4).

SHIP expression is hematopoietic restricted, where it functions to negatively regulate the phosphatidylinositol 3-kinase (PI3K) pathway (Kalesnikoff et al., 2003; Krystal, 2000). The PI3K pathway generates the important second messenger PIP₃ which regulates the association of pleckstrin homology (PH) domain containing proteins such as the survival/proliferation enhancing serine/threonine kinase Akt (also known as protein kinase B (PKB)) and PI-dependent protein kinase 1 (PDK1) with the membrane (Krystal, 2000; Rohrschneider et al., 2000). Through SHIP’s ability to hydrolyze the 5′-phosphate from PIP₃, it effectively dampens signalling downstream of PI3K.

SHIP has been demonstrated to be a master negative regulator of the immune system (Kalesnikoff et al., 2003; Krystal, 2000). Targeted disruption of SHIP in mice results in widespread hematopoietic abnormalities, including chronic, infiltrative myeloid hyperplasia in the lung (Helgason et al., 1998), enhanced mast cell degranulation (Huber
et al., 1998; Huber et al., 1998) and progressive splenomegaly, due to both extramedullary erythropoiesis and myelopoiesis (Helgason et al., 1998). The frequency of peripheral B cells is reduced as SHIP-deficient mice age because of the large number of monocyte/ MΦs which overproduce IL-6. This high level of IL-6 both inhibits B cells

**Figure 1.4 Structure and function of SHIP.** SHIP negatively regulates PI3K pathways through the hydrolysis of the 5′-phosphate of PI-3,4,5-P3 to PI-3,4-P2.

and enhances myeloid cell development (Nakamura et al., 2004). Total T cell numbers are also slightly decreased in young SHIP-deficient mice but, as the mice age, they develop greatly reduced percentages of CD4+CD8+ double positive cells and increased numbers of CD4+, CD8+ and activated T cells. Many SHIP-deficient CD4+ cells in both thymus and spleen possess a regulatory T cell (Treg) phenotype, suggesting that SHIP
restricts Treg development (Kashiwada et al., 2006). However, in mice bearing a T cell-specific deletion of SHIP this increase in Tregs is not observed, suggesting this effect is not intrinsic to the T cells (Tarasenko et al., 2007a). Rather, as evidenced by mice with a specific macrophage-granulocyte lineage deletion of SHIP that do show an activated T cell phenotype and increased numbers of Tregs (Tarasenko et al., 2007a), it is likely the whole body environment of SHIP-deficient mice that causes the T cell phenotype.

SHIP-deficient mice also suffer from severe osteoporosis owing to the action of an increased number of Paget-like, hyper-resorptive osteoclasts (Takeshita et al., 2002). Ship−/− mice display a progressive perturbation of NK cell development, characterized by an abnormally high expression of inhibitory receptors (Wang et al., 2002). The NK cell abnormalities likely play a major role in the reduced acute graft-versus-host disease (GvHD) and deficient allograft rejection that is characteristic of Ship−/− mice (Wang et al., 2002). SHIP-deficient mice also possess an increased number of Gr1+Mac1+ myeloid derived suppressor cells (MDSCs), which impair the priming of allogeneic T cells, and may therefore also play a role in suppressing GvHD (Ghansah et al., 2004). As well macrophages in Ship−/− mice tend to be skewed towards an alternatively activated macrophage (M2)‘healer’phenotype (Rauh et al., 2005).

1.13 Aims of study

SHIP is a critical negative regulator of the PI3K pathway in hematopoietic cells and, when absent, a marked increase in myelopoiesis and myeloid cell hyper-responsiveness is observed. Much investigation has focused on the role of SHIP in macrophages owing to the fact that infiltration of monocytes and macrophages into vital organs, particularly lung, are thought to contribute to the early mortality of SHIP-deficient mice. In addition, other studies have defined a role for SHIP in B cell development and function. However, little attention has been devoted to the role of SHIP in DC development or function and, as outlined above, DCs are critical for the orchestration of an appropriate immune response. Thus, with the known importance of DCs in the co-ordination of the innate and adaptive immune systems to ward off...
infection, eliminate cancerous transformations and prevent autoimmune disease, it appeared there was much insight that could be gained from studying the role of SHIP in DCs.

There are two primary systems developed for the in vitro generation of DCs from BM. Therefore, we sought to determine how SHIP deletion would impact the generation and innate activation of both GM-CSF and Flt3L-derived DCs. Specifically, we wanted to determine if SHIP: 1) regulates DC development, 2) regulates TLR-induced DC activation and 3) functionally controls TH activation and responses.

Previous studies in our laboratory have shown that Ship-/- macrophages are predominantly or solely M2 skewed (alternatively activated healer-type macrophages). Perhaps for this reason, tumour cells implanted in SHIP-deficient mice grow more rapidly than in wild-type mice. This result suggests that SHIP-deficient mice, as a whole, are less immunostimulatory. Consequently, we wanted to investigate the role of Ship-/- DCs in the development and progression of the EAE mouse model of multiple sclerosis.

Lastly, DCs are known to not only have important functions in immune activation, but also immune suppression. As such, we wanted to subject various Ship-/- DCs to activated T cells and assess their ability, relative to WT DCs, to suppress T cell proliferation. Based on the finding that Ship-/- macrophages are more immunosuppressive we hypothesized that this would also be case in DCs.

Overall, our goal over the course of these studies was to gain a more complete understanding of the role SHIP plays in the gamut of DC functions.
CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

All mice used were between 6 and 12 weeks of age. Previously described (Helgason et al., 1998) wild type (WT or +/-) and Ship--/- mice, backcrossed onto a C57Bl/6 background for at least 12 generations (provided by Dr Frank Jirik, University of Calgary, Calgary, AB) were used for the majority of experiments. Where indicated, WT and Ship--/- mice, from a colony with a mixed C57Bl/6 x 129Sv background were used. OTII transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house. Mice were initially housed in the Joint Animal Facility (JAF) of the old BC Cancer Research Centre (BCCRC) and then in the Animal Resource Centre (ARC) of the new BCCRC under specific pathogen free conditions and according to approved and ethical treatment of animal standards of the University of British Columbia. Animals were euthanized by CO2 asphyxiation at the termination of experiments.

2.2 Reagents and cytokines

*E. coli* LPS serotype O127:B8 was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC purified phosphorothioate-modified CpG-containing oligodeoxynucleotide (1826; 5'-tccatgacgttcctgacgtt-3') was purchased from Invitrogen (Burlington, ON). The dsRNA used was a synthetic analogue of polyinosine:cytosine (poly I:C) from Sigma-Aldrich (St. Louis, MO). Recombinant H18 flagellin from enteroaggregative *E. coli* strain 042 was provided by Dr. Ted Steiner (University of British Columbia). It was expressed using a pCR-NT-T7 vector with an N-terminal 6xHis tag in BL21 (DE3) pLysS (induced with IPTG) and purified using Talon cobalt resin (Clontech, Mountain View, CA). The purified flagellin was passed over polymyxin B agarose until it tested negative for endotoxin via the limulus amebocyte lysate assay. The purity and concentration of the flagellin preparation was assessed by SDS-PAGE. Peptidoglycan (PGN) from *Staphylococcus aureus* was purchased from Fluka (Buchs, Switzerland). The PI3K inhibitor, LY294002, was from Calbiochem (La Jolla, CA, USA).
Recombinant mouse (rm) cytokines (GM-CSF, Flt3L and IL-4) and tissue culture reagents were from StemCell Technologies Inc (Vancouver, BC) except for monothioglycerate (MTG) and β-mercaptoethanol (β-ME), which were from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.3 Tissue culture

2.3.1 Generation of GM-CSF ± IL-4-derived DCs

BM cells were aspirated from the femurs and tibias of mice using Hanks' Balanced Salt Solution (HBSS) containing 5% fetal calf serum (FCS) (HF). Cells were washed once and then resuspended in HF and incubated with three volumes of ammonium chloride (NH₄Cl) buffer solution (0.8%, 0.1 mM EDTA) for 5-10 min on ice. Cells were washed with HF and resuspended in IMDM containing 10% FCS, 0.00125% (v/v) MTG, 2 mM glutamine and 100 U/ml penicillin/streptomycin (hereafter referred to as mDC base). Nucleated cells (enumerated manually by counting nuclei in 3% acetic acid solution (1:50 cell dilution)) were diluted to 6x10⁵ c/ml in mDC base containing 10 ng/ml rmGM-CSF alone (GM-DCs) or with 10 ng/ml rmIL-4 (GM/IL4-DCs). Cells were seeded at 6x10⁵ c/well (1ml) in 12 well plates to which 1 ml of fresh cytokine containing medium was added on culture day 3. On days 5 and 7, half the cell-free supernatant was replaced with fresh cytokine containing medium. Non-adherent cells were harvested on either day 6 or day 8 and DCs enriched by EasySep® CD11c-PE positive selection (StemCell Technologies, Vancouver) according to the manufacturer's instructions, unless otherwise indicated. The DC purity, determined by flow cytometry enumeration of CD11c⁺ cells, was >95% after selection.

2.3.2 Generation of Flt3L-derived DCs

BM cells were harvested and prepared as above for GM-CSF ± IL4 derived cells. After NH₄Cl lysis, cells were resuspended in RPMI containing 10% FCS, 100 U/ml
penicillin/streptomycin and 50 μM β-ME (hereafter referred to as pDC base) and diluted to 1.5x10^6 c/ml to which rmFlt3L was added to a final concentration of 100 ng/ml. Cells were seeded at 4.5x10^6 c/well (3 ml/well) in 6 well plates and left for 8 days after which non-adherent cells were harvested as Flt3L-derived DCs (FL-DCs) and used in subsequent experiments.

2.3.3 Splenocyte preparation

Spleens were harvested from mice and cells extracted by mechanical disruption and passing through a 100 μm cell strainer. Red blood cells were lysed with NH₄Cl solution at a 1 volume cells: 3 volumes NH₄Cl for 5-10 min on ice and the remaining cells were washed and resuspended in the media required for experimentation.

2.3.4 Splenic DC isolation

Splenocytes were prepared as described in section 2.3.3 and the DC population enriched using EasySep® CD11c-PE positive selection (StemCell Technologies Inc.) according to the manufacturer's instructions.

2.4 Survival and expansion studies

For both Flt3L-derived or GM-CSF-derived DCs, cells were harvested on day 8 and washed three times in pDC base or mDC base, respectively, and seeded at 5x10^5 c/ml (200 μl/well) in a 96 well plate with or without the indicated concentration of cytokines. Cell survival was assessed by manually counting trypan blue excluding cells using a hemocytometer.

DC expansion was assessed by preparing both Flt3L and GM-CSF cultures as described above or in micro-cultures of 100-200 μl in 96 well plates. Non-adherent and loosely adherent cells were harvested from individual wells daily, counted on a hemocytometer and analyzed by flow cytometry for the percent of CD11c^+ cells. In
addition, we treated mice with 125 mg/kg 5-fluorouracil (5-FU) for six days to obtain a relatively synchronized population of primitive progenitors during the recovery from myeloablation. These primitive BM cells were isolated, labelled with 5 μM CFSE at 37°C for 10 min, washed extensively and then cultured and monitored for several days in culture while we tracked CFSE dilution in the CD11c+ cells by flow cytometry. At day 0 WT and Ship-/- cells were checked by flow cytometry to ensure consitent CFSE labelling and equal mean fluorescent intensity (MFI) at the commencement of the experiment.

2.5 DC maturation

To induce DC maturation the indicated concentrations of LPS, CpG, dsRNA, flagellin, PGN or Caulobacter crescentus (provided by John Smits, University of British Columbia) were added to DC cultures. Cells and supernatants were collected after 24 hrs at which point the cells were subjected to flow cytometric analysis and the supernatants frozen at -20°C for subsequent measurements of secreted cytokines by ELISA.

2.6 FACS and flow cytometry

Cell were resuspended in HBSS containing 2% FCS and 0.05% NaN₃ (HFN) and incubated with anti-CD16/32 (2.4G2) (StemCell Technologies Inc) on ice for 20 min to block FcRs before labelling. Fluorochrome conjugated or unconjugated antibodies (Table 2.1) were added at predetermined optimal concentrations for 20 min on ice. If conjugated Abs were used, cells were washed 2-3 times and resuspended in HFN. When unconjugated primary Abs were used, cells were washed and then incubated with the appropriate fluorochrome-conjugated secondary Ab. Cells were then washed 2-3 times and resuspended in HFN. Data were collected using a FACSCalibur flow cytometer using CellQuest Pro software and data were analyzed using FlowJo software.
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W–Western/immunoblotting, FC–flow cytometry
Ms–mouse, Rb–rabbit
c–cells or cell equivalents

2.7 ELISAs

Tissue culture supernatants or mouse sera were assayed for the concentration of IL-12p40, IL-6, IL-10, TNFα, IFNγ, IL-4, IL-17 and IL-2 by ELISA according to the manufacturer's instructions (BD Bioscience, Mississauga, ON). Where indicated, cytokine concentrations were also determined using a flow cytometry-based bead array for IL-6, IL-10, IL-12, TNFα, IFNγ and MCP1 (mouse inflammation kit, BD Bioscience) according to the manufacturer's instructions, with the following modifications: 5 μl of each capture bead was used per sample and the reaction was carried out in a 96-well V-bottom plate for 3 hrs. For the three washing steps, 150 μl of wash buffer was added to each well and plates centrifuged for 5 min at 1200 rpm (Beckman Coulter Allegra™ X-
12R Centrifuge, rotor SX4750). Beads were analyzed by flow cytometry using a FACSCalibur and CELLQuest software (BD) and the data analyzed using a 4-parameter logistic curve-fitting model developed by BD. The ELISA for IFNβ was adapted from two previously published protocols (Punturieri et al., 2004; Weinstein et al., 2000). Briefly, Maxisorp ELISA 96-well plates (Nalgene Nunc International, Rochester, NY, USA) were coated overnight with 0.1 μg IFNβ capture Ab (rat anti-mouse IFNβ mAb 7F-D3; Seikagaku America, Falmouth, MA, USA) in 100 μl PBS (StemCell Technologies Inc.). Wells were washed 5 times with PBS + 0.05% Tween-20 (wash buffer). Wells were blocked for 2 hrs at 23°C with 200 μl PBS + 10% heat-inactivated FCS (assay diluent) and then washed 3 times with wash buffer. Samples or standards (rmIFNβ) (100 μl) were added to each well and incubated overnight at 4°C. The wells were washed 7 times with wash buffer and then 100 μl of detection Ab (25 U/ml rabbit anti-mouse IFNβ polyclonal antibody in assay diluent; R&D Systems, Minneaplois, MN, USA) was added to each well and incubated for 2 hrs at 23°C. The wells were washed 7 times with wash buffer and 100 μl of a 1:7000 dilution of goat anti-rabbit-IgG-HRP secondary Ab (Jackson Laboratories, West Grove, PA, USA) was added to each well and the plate incubated for 1 hr at 23°C. The plate was washed 7 times with wash buffer with 1 min soaks between each wash. ELISA substrate (100 μl) (BD OptEIA TMB Substrate Reagent Set; BD Biosciences, Mississauga, ON, Canada) was added to each well and the plate incubated for 15-30 min at 23°C in the dark and then the reaction was stopped with 50 μl 2N H₂SO₄. The plate was read at 450 nm in a 96-well plate ELX808 Ultra Microplate reader (Bio-Tek Instruments Inc) and the concentration of IFNβ calculated from the standard curve.

Immunoglobulin ELISAs for anti-MOG35-55 specific Ab levels in serum were performed by coating Maxisorp ELISA plates with 100 μl/well of 10 μg/ml MOG peptide along with known concentrations of either mouse IgG or IgM dissolved in 80 mM bicarbonate buffer (pH 9.6) and bound to the plate overnight at 4°C. Wells were washed with wash buffer 3 times, and then blocked with 3% BSA in PBS for 1 hr at 23°C. Select serum dilutions (3% BSA in PBS) were added to the coated wells and
incubated overnight at 4°C. After washing with wash buffer five times, either 100 μl/well donkey anti-mouse IgG-HRP (1:7000) (Jackson Labs, West Grove, PA) or 100 μl/well (1:2500) goat anti-mouse IgM (μ-chain specific, Jackson labs, West Grove, PA) was added and incubated for 2 hrs at room temperature. For determination of IgG concentrations, plates were washed 7 times with 1 min soaks between each wash and then ELISA substrate (100 μl) (BD Bioscience, Mississauga, ON) was added to each well, incubated for 15-30 min at 23°C in the dark and then the reaction was stopped with 50 μl 2N H₂SO₄. For determination of IgM concentrations 100 μl/well (1:7000) donkey anti-goat-HRP (Jackson Labs, West Grove, PA) was added and incubated for 1 hr at 23°C. The plates were washed 7 times with 1 min soaks between each wash and ELISA substrate (100 μl) was added to each well. The plate was incubated for 15-30 min at 23°C in the dark and the reaction stopped with 50 μl 2N H₂SO₄. The plates were read at 450 nm in a 96-well plate ELX808 Ultra Microplate reader (Bio-Tek Instruments Inc) and the levels of anti-MOG Ab calculated from the standard curve.

2.8 Proliferation assays

WT or Ship-/- BM derived DCs (BMDCs) or splenic DCs were resuspended in IMDM containing 10% heat inactivated FCS, 0.00125% MTG and 100 U/ml penicillin/streptomycin (hereafter referred to as proliferation medium) and then seeded at 4x10⁴ c/well in 96 well flat bottom plates. These cultures were then stimulated with various TLR ligands (LPS at 100 ng/ml, CpG at 0.3 μM, dsRNA at 50 μg/ml, flagellin at 50 ng/ml and PGN at 100 μg/ml) and OVA₃₂₃-₃₃₉ peptide (Genscript Corp., Piscataway, NJ) at the indicated concentration and incubated for 3 hrs at 37°C. CD4⁺ T cells were isolated from OTII spleens using an EasySep® CD4-PE positive selection kit according to the manufacturer’s instructions (StemCell Technologies). CD4⁺-enriched T cells were then added to cultures at 4x10⁴ c/well to a final volume of 200 μl. Cells were incubated at 37°C for 72 hrs and labelled with ³H-thymidine (1 μCi/well) for the last 18 hrs. The contents of each well were harvested onto filtermats and the radioactivity determined
using an LKB Betaplate Harvester and Liquid Scintillation Counter (LKB Wallac, Turku, Finland).

2.9 Immune response assays

2.9.1 In vitro

WT or Ship-/- BMDCs (5x10^5 cells in proliferation medium) in 48 well plates were activated with TLR ligands (LPS at 100 ng/ml, CpG at 0.3 μM, dsRNA at 50 μg/ml, flagellin at 50 ng/ml and PGN at 100 μg/ml) and 1μg/ml OVA323-339 and then 5x10^5 CD4^+ OTII cells were added for 4 days, after which co-culture supernatants were collected.

2.9.2 In vivo

WT or Ship-/- BMDCs were incubated with TLR ligands (LPS at 100 ng/ml, CpG at 0.3 μM, dsRNA at 50 μg/ml) and 1 μg/ml OVA323-339 peptide for 16 hrs and the cells were then harvested and washed three times in proliferation medium and resuspended at 4x10^6 c/ml. Activated cells were injected intravenously (IV) into OTII transgenic mice (1x10^6 DCs (250 μl/mouse)). After 4 days, OTII mice were euthanized, their spleens removed and prepared as described in section 2.3.3. Splenocytes were resuspended in proliferation medium and cultured in 96 well flat bottom plates at 5x10^5 c/well with 1 μg/ml OVA323-339 peptide. Alternatively, CD4^+ T cells were enriched from these splenocytes and cultured with LPS-, CpG- or dsRNA-activated WT or Ship-/- BMDCs. Supernatants were collected from wells after 3 days of culture and the concentration of secreted cytokines determined by ELISA.

WT or Ship-/- mice were immunized subcutaneously (SC) with 100 μg OVA (50 μl) (Sigma-Aldrich, St Louis, MO) mixed 1:1 with complete Freund's adjuvant (Pierce Chemical, Rockford, IL) or immunized by intraperitoneal (IP) injection (100 μl) with 100 μg OVA mixed (1:1) with alum (Pierce Chemical, Rockford, IL). After 10 days, cells from draining LNs and the spleen were harvested and cultured in 96 well flat bottomed
plates at 5x10^5 c/well with 100 μg/ml OVA. Alternatively, LN and splenic CD4^+ T cells were isolated and co-cultured (1:1 ratio) with WT or Ship-/- BMDCs pre-incubated with 100 μg/ml OVA for 16 hrs. After 3 days, supernatants were collected and analyzed for secreted cytokines by ELISA.

To measure Ag uptake, BMDCs were incubated with 100 μg/ml FITC-labelled albumin at 0°C (background control) or 37°C for 2 hr. The incorporation of FITC-albumin was monitored in CD11c^+ cells by flow cytometry.

**2.10 Cell stimulations, SDS-PAGE and Western analyses**

For FL-DC stimulations, cells were harvested and washed 3 times with pDC base, enumerated and resuspended at 2.5x10^6 c/ml. Cells were then equilibrated in a 37°C water bath for 10 min before stimulating with 100 ng/ml LPS for the indicated times. Stimulation was stopped with the addition of 1 ml cold HBSS. Cells were then pelleted at 2000 rpm for 5 min in a 4°C microfuge (Biofuge pico, Heraeus). Cell pellets were lysed in 1x SDS sample buffer (8.5% (v/v) glycerol, 0.5% (w/v) SDS and 0.71 M β-ME).

For Western blot analysis, equal numbers of cells or cell equivalents (total cell lysates) for each time point were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using the methods and equipment included with the Protean II™ electrophoresis system (Bio-Rad Laboratories, Mississauga, ON). Size fractionated proteins from the gels were electrophoretically transferred to Immobilon™ polyvinylidene difluoride (PVDF) membranes using a Trans Blot Cell™ transfer system (Bio-Rad) as per the manufacturer's instructions and the PVDF membranes were blocked with 5% BSA (w/v) in phosphorylation solubilization buffer (PSB, 50 mM HEPES, pH 7.4, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 4 mM EGTA) for 1 hr at 23°C or overnight at 4°C. Membranes were then incubated with the appropriate primary Ab diluted as indicated (Table 2.1) in 2% BSA and 0.1% azide (v/v) in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM KCl and 0.01% Tween-20) for 1 hr at 23°C or overnight at 4°C. Blots were then washed 5 x 5 min with TBST (no azide) followed by incubation for
45 min at 23°C with 1:10,000 dilution (in TBST) of the appropriate HRP-conjugated secondary Ab. Blots were then washed another 5 x 5 min and detected using Western Lightning Enhanced Chemiluminescence (ECL) reagent (Perkin Elmer, Boston, MA) and Kodak X-Omat Blue film.

2.11 RNA extraction, RT-PCR and qPCR

Total RNA was prepared from cells using TRIzol® Reagent (Invitrogen, Burlington, ON) and genomic DNA contaminants were removed using a TURBO DNA-free™ kit (Ambion®, Applied Biosystems, Foster City, CA) according to each manufacturer's instructions. cDNA synthesis was performed using M-MLV RT (Invitrogen) with an oligo (dT)₁₈ primer. The reactions were performed as per the manufacturer's instructions with the following modification: the reactions were linearly scaled to a 25 µl total volume. PCR amplifications were performed using the Phusion® High-Fidelity DNA Polymerase kit (Finnzymes, Espoo, Finland). Each reaction was performed in a 25 µl total volume consisting of 1 µl template, 16.5 µl PCR-H₂O, 5 µl of 5x Phusion HF buffer (containing 7.5 mM MgCl₂), 0.75 µl DMSO, 0.25 µl Phusion High-Fidelity DNA Polymerase (2 U/µl), 0.5 µl of a 10 µM forward primer solution, 0.5 µl of a 10 µM reverse primer solution and 0.5 µl of a dNTP (10mM each) solution (Fermentas, Burlington, ON). Internal control amplification reactions using specific primers for a 541 bp GAPDH fragment amplicon were performed (5'-TTAGCCCCCCTGGCCAAGG and 5'-CTTACTCCTTGGAGGCCATG). Amplification of mouse TLRs (mTLRs) were performed with primers purchased from Invivogen (Burlington, ON), designed to yield the amplicon sizes shown in Table 2.2.

Table 2.2 RT-PCR primers used to amplify mTLR transcripts.

<table>
<thead>
<tr>
<th>mTLR primer pair</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTLR1</td>
<td>309 bp</td>
</tr>
<tr>
<td>mTLR2</td>
<td>400 bp</td>
</tr>
<tr>
<td>mTLR3</td>
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<td>mTLR5</td>
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## mTLR primer pair and Product Size

<table>
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<tr>
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<th>Product Size</th>
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<td>mTLR8</td>
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<td>mTLR9</td>
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</table>

RT-Primers for mTLRs were purchased from Invivogen (Version # 07H09-SV). The expected product size for each mTLR primer pair is given in base pairs (bp).

PCR amplifications were performed at 98°C for 75 sec, followed by 35 cycles of 98°C for 15 sec, 58°C or 63°C for 20 sec, 72°C for 30 sec followed by a final step of 72°C for 10 min. All reactions were performed using a GeneAmp® PCR System 9700 thermo cycler (Perkin Elmer Applied Biosystems, Waltham, MA).

For IL-2 quantitative PCR (qPCR), RNA was extracted using the RNeasy mini kit (QIAGen, Mississauga, ON) according to the manufacturer's instructions. 1µg RNA, treated with DNase I (Invitrogen), was used as a template for reverse transcription using Superscript III (Invitrogen) according to the manufacturer's instructions. Control reactions were performed with no enzyme to ensure there was no DNA contamination. IL-2 transcripts were detected by qPCR in comparison to a GAPDH internal control amplicon. cDNA was diluted 10-fold for IL-2 and 10,000-fold for GAPDH amplification reactions. 3 µl of the dilute templates and 0.2 µM of each primer were used in each 10 µl reaction with 1 x FAST SYBR green mix (Applied Biosystems). Reactions were run on an Applied Biosystems 7500 Fast real-time PCR machine with 20 sec initial denaturation at 95°C followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C. Agarose gel electrophoresis and dissociation analysis were performed to ensure each primer pair yielded only one product. Data were analyzed using the ΔC(T) method of relative quantitation so the data are presented as IL-2 expression relative to GAPDH expression. The primers used for IL-2 were IL-2-fwd 5’-GACCTCTGCGGCATGTTCT-3’ and IL-2-rev 5’-CCACCACAGTTGCTGACTCA-3’. GAPDH primers were GAPex6F 5’-GACTTCAACAGCAACTCCCAC-3’ and GAPex7R 5’-TCCACCACCTGTTGCTGTA-3’.
2.12 Induction of experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was induced in 6-10 week old female WT and Ship-/- mice (F2 mixed C57Bl/6 x 129Sv background). The injected MOG peptide emulsion was prepared by adding equal volumes of peptide derived from mouse myelin oligodendrocyte glycoprotein (MOG35-55 (MEVGWYRSPFRSRVSRRVHGK, synthesized at the UBC Peptide Core Facility (Vancouver, BC)) and CFA containing 4 mg/ml heat-inactivated Mycobacterium tuberculosis H37RA (Difco Labs Inc., Lawrence, KS). Each mouse received a total of 100 μg peptide and 400 μg Mycobacterium tuberculosis, administered as three SC injections totalling 100 μl volume. Mice also received Pertussis toxin (200 ng/mouse/IV injection on days 0 and 2). Mice were observed daily for clinical signs of disease and were scored on a clinical scale as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, paraplegia or tetraplegia; and 5, death or mouse euthanized due to severe morbidity.

2.13 Peptide recall assays

Spleens and LNs were removed from mice 10 days after immunization with MOG35-55. The tissues were homogenized and passed through a nylon 100 μm cell strainer followed by NH4Cl red blood cell lysis. The remaining cells were washed twice in HF, then resuspended in proliferation medium and plated (5x10^5 c/well) in 96 well flat bottom plates with the indicated concentration of MOG35-55 for 72 hrs at 37°C. 3H-thymidine (1 μCi/well) was added for the last 18 hrs at which point the contents of each well were harvested onto filtermats and the incorporated radioactivity counted using an LKB Betaplate Harvester and Liquid Scintillation Counter (LKB Wallac, Turku, Finland). For measurement of cytokine production from spleen cells, prepared splenocytes were plated in 48 well plates (2.5x10^6 c/well) with the indicated concentration of MOG35-55 and the culture supernatants harvested after 72 hrs. Cytokine concentrations in the supernatant were determined by ELISA.
2.14 Determination of microglial content in the brain and spinal cord

Brains and spinal cords were isolated from PBS-perfused mice and the tissues minced in HBSS containing 400 U/ml collagenase IV, incubated for 30 min at 37°C and cells separated by passing through a 70 μm nylon filter. Single cell suspensions were washed with HBSS and pelleted by centrifugation at 1200 rpm for 5 min at 4°C (Eppendorf centrifuge 5810, swinging bucket, A-4-62 rotor). Cells were then resuspended in 2-5 mls of a 30% percoll solution (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and leukocytes isolated by centrifugation at 1200 rpm at 23°C (Eppendorf centrifuge 5810, swinging bucket, A-4-62 rotor). Leukocytes were recovered from the pellet, labelled with specific Abs and analyzed by flow cytometry. In some experiments, microglia (CD45⁺CD11b⁺ cells) were also sorted by FACS.

2.15 Arginase assay

Arginase activity was assessed indirectly by measuring the concentration of urea generated from the hydrolysis of L-arginine (Corraliza et al., 1994; Schimke, 1962). Cells were lysed for 10 min in a 1:1 mixture of 0.1% Triton X-100 and 25 mM Tris-HCl containing the protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml) and PMSF (0.5 mM) (lysis buffer). The total protein concentration of lysates was determined using a Bradford assay (Bio-Rad) and 2.5 μg of protein was diluted in lysis buffer to 100 μl and used for subsequent arginase activity determinations. The lysate was added to 10 μl of 10 mM MnCl₂ at 55°C for 10 min to activate endogenous arginase, and then substrate (ie., 100 μl of 0.5 M L-arginine (pH 9.5)) was added. The reaction was allowed to proceed for 1 hr at 37°C. Next, 800 μl of 10% H₂SO₄:30% H₃PO₄:70% H₂O was added to stop the reaction and finally 40 μl of 9% (w/v in absolute ethanol) α-isonitrosopropiophenone was added and incubated at 100°C for 30 min to detect urea. Absorbance of the samples at 550 nm was compared to a urea standard curve to calculate arginase activity.
2.16 Nitric oxide assay

Nitric oxide (NO) production was determined indirectly by measuring the accumulation of nitrite (NO$_2^-$), one of two primary, stable breakdown products of NO, in the tissue culture supernatant using a modification of the Griess assay ((Griess, 1879; Stuehr and Nathan, 1989). Briefly, 50 μl of supernatant was sequentially incubated with equal volumes of 1% sulfanilamide in 2.5% phosphoric acid and 0.1% phenyl-napthylenediamine dihydrochloride in 2.5% phosphoric acid at 23°C. After 5 min, the absorbance of samples at 570 nm was determined and NO$_2^-$ concentration calculated by comparison to a NaNO$_2$ standard curve.

2.17 T cell suppression assay

This assay was performed based on the protocol described by Thornton and Shevach (Thornton and Shevach, 1998) with a few modifications. WT or Ship-/- BMDCs or splenic DCs were plated at 5x10$^4$ c/well in a 96 well flat bottom plate and serial 1:2 dilutions performed down to 3.125x10$^3$ c/well. Prepared splenocytes were stimulated with 0.5 μg/ml αCD3 and 2.5 μg/ml αCD28 (eBioscience, San Diego, CA) (to stimulate T cell proliferation) and added (2x10$^5$ c/well) alone or to the DC-containing wells in 200 μl total volume. Cells were incubated at 37°C for 72 hrs, with ³H-thymidine (1μCi/well) added for the last 18 hrs. The contents of each well were then harvested onto filtermats and counted using an LKB Betaplate Harvester and Liquid Scintillation Counter (LKB Wallac, Turku, Finland). In some experiments, blocking Abs to CTLA-4 (BD Biosciences, Missisauaga, ON), Mac-1 and LFA-1 (Dr. Fumio Takei, University of British Columbia), IL-4 (eBioscience, san Diego, CA), TGFβ, IL-13, IL-6, IL-10, IFNγ (R&D Systems, Minneapolis, MN) and inhibitors Bec (Calbiochem, San Diego, CA), PTIO (Cayman Chemical, Ann Arbor, MI), L-NMMA, N-acetylcysteine (NAC), superoxide dismutase (SOD) (Sigma-Aldrich, St. Louis, MO) and exiguamine A (Dr. Ray Anderson, Vancouver, BC) were added to DC-containing wells just prior to the addition of the activated splenocytes. Percent suppression of proliferation was calculated as follows:
Relative percent suppression of proliferation was calculated as

\[ \left( 1 - \frac{\text{proliferation with DCs with inhibitor}}{\text{proliferation without DCs with inhibitor}} \right) \times 100 \]

All conditions were performed in triplicate. In parallel, similar assays were carried out in 48-well (600 μl total volume) plates to allow supernatant collection and analysis by ELISAs. Transwell experiments were conducted in 96 well 0.4μm transwell plates (Corning, Lowell, MA) in 250 μl total volume. DCs were plated in the bottom chamber and stimulated splenocytes plated in the top chamber. The 0.4 μm semi-permeable membranes that separate the upper and lower chambers allow diffusion of soluble materials but not cell migration. Control conditions, consisting of wells containing only activated splenocytes in the top chamber and media in the bottom chamber were also performed.

2.18 Statistical analysis

Statistical significance was calculated using a two-tailed unpaired student \( t \) test using Microsoft excel. Differences were considered significant when \( p < 0.05 \).
CHAPTER 3: SHIP NEGATIVELY REGULATES GM-CSF-DERIVED DENDRITIC CELL GENERATION BUT POSITIVELY REGULATES TLR-INDUCED MATURATION AND FUNCTION

3.1 Introduction

Dendritic cells (DCs) are bone marrow (BM)-derived leukocytes that are specialized for the uptake, processing and presentation of antigens (Ags). After encountering Ag DCs migrate to secondary lymphoid organs where they activate T cells and direct an adaptive immune response (Banchereau et al., 2000; Mellman and Steinman, 2001). Upon encountering a pathogen, DCs generate an innate immune response consisting of cytokine production and initiation of a cellular or humoral immune response through T<sub>H</sub> cell polarization (Boonstra et al., 2003; Kapsenberg, 2003; Mazzoni and Segal, 2004). Recognition of pathogens occurs in part through stimulation of Toll-like receptors (TLRs) expressed by DCs (Pasare and Medzhitov, 2004). Engagement of TLRs results in the activation of either the MyD88-dependent or – independent pathways, or both, depending on the particular TLR activated, (Akira et al., 2006; Beutler, 2004; Hoebe and Beutler, 2008; Iwasaki and Medzhitov, 2004). TLR-ligand association initiates the process of maturation (resulting in upregulation of MHCII and costimulatory molecules) as well as inducing cytokine production (in particular IL-12, a key inducer of T<sub>TH1</sub> responses) (Pasare and Medzhitov, 2004). Several signalling pathways have been implicated in the regulation of these events. One is the PI3K pathway, which is negatively regulated, in part, by the lipid phosphatase SHIP (Kuo et al., 2006; Sly et al., 2007). However, SHIP's contribution to the generation, maturation and innate activation of DCs is poorly understood.

Class I PI3Ks are eukaryotic lipid kinases that are activated by a wide array of extracellular stimuli and generate the critical second messenger phosphatidylinositol-3,4,5 trisphosphate (PIP<sub>3</sub>) (Koyasu, 2003; Vanhaesebroeck et al., 2001). PIP<sub>3</sub>, in turn, attracts signalling proteins that contain plekstrin homology (PH) domains with a preferential affinity for PIP<sub>3</sub>, for example, the serine/threonine kinase Akt, which when
phosphorylated/activated, typically enhances cell survival and proliferation (Koyasu, 2003; Vanhaesebroeck et al., 2001). Although numerous studies have shown that PI3K regulates TLR signalling, it remains uncertain if it is a positive or negative regulator. However, the prevailing hypothesis is that PI3K is a negative regulator of TLR pathways (Hazeki et al., 2007). The majority of these studies have focused on the role of PI3K in TLR-induced activation of macrophages (MΦs), with only a small subset of these studies investigating its role in DC functions. One study within this small subset showed that both splenic and BM-derived DCs from mice lacking the p85α regulatory subunit of class IA PI3Ks produced more IL-12 upon stimulation with each of the TLR ligands LPS, peptidoglycan (PGN) and CpG (Fukao et al., 2002). In addition, treatment of wild type (WT) DCs with the PI3K inhibitor, wortmannin, resulted in increased LPS-induced IL-12 production. These results suggest that PI3K is a negative regulator of IL-12 production by DCs (Fukao et al., 2002; Fukao and Koyasu, 2003).

SHIP negatively regulates the PI3K pathway through hydrolysis of the 5' phosphate from PIP3, generating PI-3,4-P2 (Krystal, 2000; Rohrschneider et al., 2000). Several groups have reported a role for SHIP in MΦ development and innate activation (An et al., 2005; Fang et al., 2004; Parsa et al., 2006; Sly et al., 2009; Sly et al., 2004). However, only one article exists on the role of SHIP in DCs (Neill et al., 2007). In this chapter we examine the contribution of SHIP to GM-CSF-derived DC (GM-DC) generation and innate activation. Based on evidence that PI3K is a negative regulator of TLR activation in DCs, we hypothesized that SHIP would counter this activity and act as a positive regulator of TLR-induced DC activation.

3.2 Results

3.2.1 Expression of negative regulators of the PI3K pathway in WT and Ship−/− DCs

The PI3K pathway is important in immune cells and perturbation of this pathway has a dramatic effect on the ability of these cells to respond to infectious agents. Thus, the activity of the PI3K pathway must be carefully regulated to avoid too weak or too strong
a response. As such, it is critical that the PI3K pathway be tightly regulated by both positive and negative modulators. To determine if other negative regulators of the PI3K pathway are upregulated in DCs when SHIP is absent, we compared the levels of SHIP2 and PTEN (two well established negative regulators of the PI3K pathway) in WT and Ship-/- GM-DCs by Western analysis. As shown in Fig 3.1, we found that WT and Ship-/- DCs express the comparable levels of PTEN and SHIP2, indicating that loss of SHIP does not result in a compensatory increase in these two lipid phosphatases.

**Figure 3.1 Expression of negative regulators of the PI3K pathway in WT and Ship-/- GM-CSF-derived DCs.** WT and Ship-/- DCs were purified from day 8 cultures and probed with specific antibodies to SHIP2, SHIP1 and PTEN and subjected to Western blot analysis. GAPDH serves as a loading control. Data shown are representative of at least three independent experiments in which comparable levels of SHIP2 and PTEN were seen in WT and Ship-/- GM-DCs.

### 3.2.2 Enhanced expansion of DCs from Ship-/- BM precursors *in vitro*

It has previously been reported that SHIP deletion results in enhanced proliferation of macrophage and granulocyte lineages, both *in vitro* and *in vivo*. This is illustrated by elevated levels of granulocyte-macrophage colony forming units (CFU-GM) present in the spleen and BM of SHIP-deficient mice, likely because of their hyper-responsiveness to IL-3, IL-6, SF, GM-CSF and M-CSF (Helgason et al., 1998; Liu et al., 1999). Since DC progenitors may also be similarly hyper-responsive to growth-promoting factors, we determined if BM precursors harvested from Ship-/- mice generate more DCs. Freshly isolated BM cells from WT and Ship-/- mice were cultured with
various concentrations of GM-CSF. The non-adherent and loosely adherent cells from both cultures showed the same characteristic DC morphology after six days in culture and the DCs from both sets expressed similar levels of cell surface CD11c and CD11b (characteristic of DCs) by flow cytometry (data not shown). However, we found that Ship-/- BM generated a higher frequency of CD11c+ cells in culture, in keeping with a previous report by Neill et al. (Neill et al., 2007). However, in contrast to Neill et al, we found that total cell numbers from Ship-/- DC cultures were consistently higher than the total cell numbers from WT cultures and, when combined with the increased percentage of DCs, generated approximately 50% more DCs at all concentrations of GM-CSF tested (Fig. 3.2A, left panel). Of note, after eight days in culture with 5 ng/ml GM-CSF, Ship-/- BM cultures had reached approximately 75% of their maximum number of DCs produced while WT cultures had reached only 55% of their maximum potential. Time course studies using 10 ng/ml GM-CSF revealed that more Ship-/- DCs were generated from BM on each day of culture (Fig 3.2A, right panel). Importantly, by culture day 5, the number of DCs generated from Ship-/- BM had plateaued while DCs from WT BM had only reached approximately 50% maximum production. These results suggest that Ship-/- DC precursors are hypersensitive to GM-CSF compared to WT DC precursors. However, interpretation of these results is complicated by the fact that SHIP knockout mice are reported to have four-fold higher numbers of BM DC progenitors than WT mice (Neill et al., 2007). Therefore, in order to address this complication, we next examined the proliferation of individual cells, independent of precursor frequency, by tracking the division of CD11c+ DCs using CFSE dilution. To do this experiment, we treated mice with 125 mg/kg 5-fluorouracil (5-FU) for six days to obtain a relatively synchronized population of primitive progenitors during the recovery from myeloablation. These primitive BM cells were isolated, labelled with 5 μM CFSE and then cultured and monitored for several days in culture while we tracked CFSE dilution in the CD11c+ cells. Our results from this experiment suggest that the generation of CD11c+ cells from Ship-/- BM precursors was indeed more rapid than WT BM precursors as indicated by a more rapid dilution of CFSE (ie., more cell divisions) in culture (Fig 3.2B). The Ship-/- BM cells showed not only an increased rate of cell division, but also accelerated differentiation into CD11c+ cells. The frequency of CD11c+ cells was higher in Ship-/-
Figure 3.2 Enhanced \textit{in vitro} expansion of GM-CSF-derived \textit{Ship}-/- DC bone marrow precursors. CD11c$^+$ DCs were enumerated by hemocytometer and flow cytometry. (A) Left panel, BM cells isolated from WT (+/+) (■) and SHIP-deficient (-/-) (Δ) mice were cultured in 96 well plates as a 1/10 microculture of the standard derivation conditions. BM was cultured with concentrations of GM-CSF ranging from 0 to 50 ng/ml for 8 days. Right panel, growth kinetics were determined by culturing cells with 10 ng/ml GM-CSF using standard culture conditions described in the Materials and Methods and harvesting and counting daily for 8 days. (B) BM cells isolated from 5-FU-treated mice were labeled with CFSE and cultured in 10 ng/ml GM-CSF. On days 3, 4 and 5, CFSE dilution was determined in the CD11c$^+$ population by flow cytometry. The mean fluorescence intensity (MFI) for the CD11c$^+$ cells is indicated. (C) Differentiation kinetics were determined daily during 8 days of culture with 2, 10 or 50 ng/ml GM-CSF by measuring the percentage of CD11c$^+$ cells. (D) DCs were purified from day 8 cultures and washed thoroughly and cultured in mDC base with or without 10 ng/ml GM-CSF and survival assessed by trypan blue exclusion. All assays were performed in triplicate and results displayed are the mean ± SEM. Data shown are representative of two or three independent experiments. * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. \textit{Ship}-/- DCs)
cultures, particularly during the earlier days of culture (days 3 and 4) and with low concentrations of GM-CSF (Fig 3.2C).

Since higher numbers of Ship-/- DCs from whole BM cultures could also be the result of prolonged survival (rather than enhanced proliferation) in response to growth factors. Therefore, we tested the survival of differentiated (culture day 8) WT and Ship-/- DCs in the presence or absence of GM-CSF. Using trypan blue exclusion to determine viability, we found that Ship-/- DCs did indeed display enhanced survival. Specifically, Ship-/- DCs survived better than WT DCs, both in the presence and absence of GM-CSF. Strikingly, the survival of Ship-/- DCs in the absence of GM-CSF surpassed that of the WT DCs in the presence of GM-CSF (Fig 3.2D). Thus, in addition to enhanced proliferative response to GM-CSF, Ship-/- DCs display enhanced growth factor-independent and growth factor-induced survival.

3.2.3 Enhanced expansion of DCs in Ship-/- mice

To determine if the increased generation of DCs from Ship-/- BM in vitro was mirrored by increased numbers in vivo, we enumerated splenic DCs by flow cytometry. The percentage of CD11c+ splenocytes from Ship-/- mice was higher than age and sex matched WT littersmates (5.0 ± 1.03% in WT vs. 8.1 ± 0.87% in Ship-/-; n=3). In addition, since the total number of splenocytes is also higher in Ship-/- mice (177 ± 32.7 x 10^6 in WT vs. 204 ± 56.2 x 10^6 in Ship-/-; n=3), there is an overall two-fold higher total number of DCs in the spleen of SHIP-deficient mice (8.86 ± 2.3 x 10^6 in WT vs. 16.3 ± 3.9 x 10^6 in Ship-/-; n=3). Further analysis of the splenic CD11c+ populations revealed a slight decrease in the percentage of CD11b+ cells in Ship-/- mice. However, Ship-/- splenic DCs contained a population of CD11b\textsuperscript{high} as well as CD11b\textsuperscript{mid} DCs, resulting in increased mean fluorescence intensity (MFI) for the entire population (Fig 3.3). Ship-/- DCs also had a concomitant increase in CD8\alpha+ DCs and a decrease in the number of B220+ plasmacytoid DCs. In addition, the DCs found in the spleen of SHIP-deficient mice showed a less mature phenotype, as indicated by a significant reduction in the percentage of MHCII+ cells as well as a lower overall level of MHCII (Fig 3.3).
Figure 3.3 Analysis of splenic DC populations. Spleens were isolated from age and sex-matched littermates. Cell suspensions were prepared as described in Materials and Methods. Flow cytometric analysis of CD11c+ splenic DCs were analysed for the expression of the indicated surface molecule. □ Isotype Control; mean fluorescent intensity (MFI) and the percentage positive cells are indicated on the histograms. Data shown are representative of three independent experiments.

3.2.4 Impaired maturation of Ship-/- DCs after TLR activation

The development of an appropriate DC response to an inflammatory agent depends on its maturation - typically induced by exposure to Ags and innate immune stimuli. To examine the ability of Ship-/- DCs to mature following stimulation with TLR ligands, we treated WT and Ship-/- GM-DCs with or without 100 ng/ml LPS, 0.3 μM CpG or 50 μg/ml dsRNA for 24 hrs at 37°C. We found that the in vitro derived Ship-/- DCs, like in vivo derived Ship-/- splenic DCs, were far less mature, even before TLR ligand-induced maturation. For example, as shown in Fig 3.4, Ship-/- CD11c+ cells expressed little to no MHCII or CD40, while non-stimulated WT CD11c+ cells expressed high levels of these maturation markers. As well, in response to all three TLR ligands, we observed only a slight increase in the expression of CD40 and MHCII in Ship-/- cells,
Figure 3.4 GM-CSF-derived *Ship*-/- DCs fail to mature appropriately in response to innate immune activation. Day 8 DC cultures were stimulated with LPS, CpG and dsRNA for 24 hrs. The maturation of CD11c⁺ cells in media-treated (blue line) and TLR ligand-treated (red line) cultures was assessed by expression of CD40, CD80, CD86 and MHCII (isotype control, grey line) by flow cytometry. The increase in percent positive cells from control to stimulated is indicated by the region markers. Data shown are representative of three independent experiments.
compared to WT DCs (Fig 3.4 and Table 3.1). The impaired ability of Ship-/- DCs to respond to these TLR ligands was not due to a lower expression of the cognate receptors.

Table 3.1 MFI of MHCII and costimulatory molecules for unstimulated and TLR stimulated WT and Ship-/- DCs

<table>
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<th>unstim</th>
<th>LPS</th>
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<th>dsRNA</th>
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<tr>
<td></td>
<td>WT</td>
<td>Ship-/-</td>
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<td>Ship-/-</td>
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</table>

The LPS receptors, TLR4 and CD14 were expressed at comparable levels in WT and Ship-/- GM-DCs, as assessed by flow cytometry (Fig 3.5A). Similarly, the levels of TLR3, TLR4 and TLR9 mRNA, as assessed by RT-PCR (Fig 3.5B) were comparable in WT and Ship-/- GM-DCs. In addition, we found that Ship-/- and WT GM-DCs express similar levels of TLRs 1, 2, 6, 7, and 8 mRNA. Although only barely detectable levels of TLR5 were found in WT and Ship-/- DCs, these cells were still capable of responding to flagellin stimulation (Fig 3.6).
Figure 3.5 WT and Ship-/- DCs express similar levels of TLRs. (A) The expression of TLR4 and CD14 was determined for the CD11c+ population of day 8 GM-CSF-derived cultures (black line). The gray area indicates isotype control Ab labelling. The MFI of the CD11c+ cells is indicated. (B) Total RNA was isolated from day 8 WT and Ship-/- DCs and the levels of TLR transcripts determined using RT-PCR using 100 ng of template. A water control (H2O) was used to rule out the presence of contaminating RNA or DNA in the buffers. Data shown are representative of at least three independent experiments.
3.2.5 TLR-induced cytokine secretion is altered in Ship−/− DCs

In addition to up-regulating co-stimulatory molecules on DCs, innate activation also plays an important role in shaping the adaptive immune response through the production of cytokines. For example, DC secreted IL-12, IL-6 and TNFα play important roles in the regulation of immune responses. We studied the secretion of these cytokines, as well as the anti-inflammatory cytokine IL-10, by ELISA after DC stimulation with a variety of TLR ligands. Over a wide range of LPS, CpG, dsRNA, flagellin and PGN concentrations, Ship−/− GM-DCs secreted a lower level of IL-12p40 (Fig 3.6, top panels). This is consistent with the impaired maturation of Ship−/− DCs prior to and following LPS, CpG and dsRNA stimulation. We conclude that the reduced maturation of Ship−/− DCs correlates with a significant impairment in IL-12 production by these cells. In addition to these results, we found that Ship−/− DCs tended to produce lower levels of IL-10 in response to CpG, dsRNA and LPS. Although this trend was consistent for LPS stimulation (at doses tested) we note that only the results for CpG and dsRNA are statistically significant (Fig 3.6, bottom panels). In contrast, IL-6 and TNFα production were enhanced in Ship−/− DCs in response to LPS, dsRNA, flagellin and PGN, but not CpG stimulation (Fig 3.6, middle panels). This result was significant at higher doses of ligand.
Figure 3.6 *Ship-*/- GM-CSF-derived DCs produce less IL-12 after TLR activation.* GM-CSF-derived DCs were purified from day 6 cultures and treated with the indicated concentration of LPS, CpG, dsRNA, flagellin and PGN for 24 hrs at 37°C. Supernatants were collected and assayed for IL-12, IL-6, TNFα and IL-10 by ELISA. Results are mean ± SEM of two to four independent experiments performed in duplicate. IL-10 was not detectable in response to flagellin stimulation. (■) WT DC supernatants, (□) SHIP-/- DC supernatants. * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. Ship-/- DCs)
LPS (ng/ml) CpG (μM) dsRNA (μg/ml)

IL-12 (pg/ml) +/+ -/-

IL-6 (pg/ml) 0 100 1000 10000

TNFα (pg/ml) 0 100 1000 10000

IL-10 (pg/ml) 0 100 1000 10000

IL-6 (pg/ml) 

TNFα (pg/ml) 

IL-10 (pg/ml) 

LPS (ng/ml) 0 100 1000 10000

CpG (μM) 0 100 1000 10000

dsRNA (μg/ml) 0 100 1000 10000
flagellin (ng/ml) PGN (μg/ml)

IL-12 (pg/ml)

IL-6 (pg/ml)

TNF-α (pg/ml)

IL-10 (pg/ml)

Not Detectable

flagellin (ng/ml) PGN (μg/ml)
3.2.6 GM-CSF-derived Ship-/- DCs have an impaired ability to induce Ag-specific T cell proliferation in vitro

Since one of the primary functions of activated DCs is to induce Ag-specific T cell proliferation and activation, we next assessed the relative function of WT and Ship-/- DCs in vitro. Splenic CD4+ T cells isolated from OTII transgenic mice (all T cells possess a TCR that recognizes the ovalbumin peptide, OVA323-339 presented by MHCII) were co-cultured with BM-derived in vitro matured (day 8) WT or Ship-/- GM-DCs that were loaded with OVA323-339 peptide and activated with a TLR-ligand for 72 hrs. In vitro proliferation of T cells in the co-culture was determined by 3H-thymidine incorporation. In these experiments we found that induction of Ag-specific T cell proliferation was greatly reduced in response to Ship-/- GM-DCs stimulated with LPS, CpG, dsRNA, flagellin and PGN-treated compared to WT GM-DCs (Fig 3.7). We conclude that the deficient naïve and TLR-ligand induced maturation of Ship-/- GM-DCs results in impaired activation of Ag-specific T cell proliferation in vitro.

Figure 3.7 GM-CSF-derived Ship-/- DCs have a reduced ability to induce Ag-specific T cell proliferation. CD4+ T cells were isolated from OTII transgenic mice and cultured with TLR-activated DCs with the indicated concentration of OVA323-339. T cell proliferation was determined by 3H-thymidine incorporation. Results displayed are the mean ± SEM of triplicate determinations. Results are representative of at least three independent experiments.
3.2.7 Reduction of T\textsubscript{H}1 responses by GM-CSF-derived \textit{Ship}-/- DCs

DCs play a pivotal role in determining the T\textsubscript{H}1/T\textsubscript{H}2 characteristics of an immune response. Therefore, to further explore the aberrant phenotype and functions observed in \textit{Ship}-/- DCs, we next tested the character of the T\textsubscript{H}1 response they generated \textit{in vitro} and \textit{in vivo}. OTII transgenic mouse CD4\textsuperscript{+} T cells were activated \textit{in vitro} by TLR ligand-treated DCs loaded with OVA peptide. Supernatants of the co-culture were collected and assayed for IFN\textgamma and IL-4 by ELISA. The production of IFN\textgamma was significantly reduced when T cells were co-cultured with \textit{Ship}-/- compared to WT DCs. The result was the same regardless of the TLR ligand used to stimulate the DCs, except in the case of PGN where no difference between the WT and \textit{Ship}-/- DC function was detected (Fig 3.8A). IL-4 production was not detectable in any of the cultures. To study T cell polarization \textit{in vivo}, we intravenously injected OVA peptide-loaded and LPS-, CpG- or dsRNA-activated WT or \textit{Ship}-/- GM-DCs into OTII transgenic mice. The T\textsubscript{H}1 responses were measured four days later by culturing total splenocytes isolated from DC-immunized mice with OVA peptide or by isolating CD4\textsuperscript{+} T cells and co-culturing them with LPS-, CpG- or dsRNA-activated OVA peptide-loaded WT or \textit{Ship}-/- GM-DCs. Similar to the results \textit{in vitro}, we found that the production of IFN\textgamma was significantly less in both the whole splenocytes and the CD4\textsuperscript{+} T cells from OTII mice immunized with \textit{Ship}-/- DCs (Fig 3.8B). The production of IL-4 was only detectable in splenocyte cultures, with \textit{Ship}-/- immunized OTII splenocytes producing significantly less IL-4 (WT, 157 ± 8.7 pg/ml; \textit{Ship}-/- 78.4 ± 2.7 pg/ml).

In another set of experiments, we examined the immune responses of WT and SHIP-deficient mice by immunizing them with whole OVA protein mixed with an adjuvant - either complete Freund's adjuvant (CFA) (T\textsubscript{H}1-biasing) or alum (T\textsubscript{H}2-biasing). We found that \textit{Ship}-/- splenocytes and lymph node (LN) cells produce less IFN\textgamma when immunized with OVA in CFA (Fig 3.8C, left panel), whereas the production of IL-4 was increased in splenocytes from \textit{Ship}-/- mice immunized with OVA in alum (Fig 3.8C, right panel). To ensure that these cytokine-production results accurately reflect the T\textsubscript{H}1-proclivity and not simply differences in the number of T cells in LN and spleens, we next
Figure 3.8 GM-CSF-derived Ship-/- DCs induce reduced Th1 responses in vitro and in vivo. (A) CD4+ OTII T cells were cultured with OVA323-339 peptide-loaded TLR-activated WT or Ship-/- DCs and supernatants collected after 4 days for ELISA. (B) OVA323-339 peptide-loaded LPS, CpG or dsRNA-activated WT or Ship-/- DCs were i.v. injected into OTII transgenic mice. Splenocytes were collected 4 days later and cultured with 1µg/ml OVA323-339 peptide, or CD4+ T cells were purified and cultured with LPS-, CpG- or dsRNA-stimulated OVA323-339 peptide-loaded WT or Ship-/- DCs (CD4/DC). Supernatants were collected after 4 days of co-culture and subjected to cytokine ELISA. (C) WT or SHIP-deficient mice were immunized with OVA in CFA by s.c. injection (left panel) or OVA in alum by IP injection (right panel). After 10 days, cells from the spleen and lymph nodes were isolated and stimulated with 100µg/ml OVA or CD4+ cells were isolated and cultured with OVA-loaded WT or Ship-/- DCs (CD4/DC). Supernatants were collected 3 days later and subjected to cytokine ELISA (D) WT and Ship-/- DCs were incubated with FITC-albumin and the uptake at 4°C (grey area) and 37°C (black line) was monitored by flow cytometry. * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. Ship-/- DCs)
A

B

C

D

WT DCs

Ship-/- DCs

FITC

FITC

# cells
isolated CD4+ T cells from OVA-immunized WT and SHIP-deficient mice. Isolated splenic and LN CD4+ T cells were then co-cultured with DCs pulsed overnight with OVA. Consistent with our in vivo immunization results, purified LN and splenic CD4+ T cells from Ship-/- mice produced significantly less IFNγ (Fig 3.8C, left panel) in response to OVA-presenting DCs. IL-4 production was below detection levels in both WT and Ship-/- cells. Notably, Ship-/- DCs are fully competent in the capture of Ag, since Ship-/- DCs had a similar, if not an enhanced, ability to take up FITC-labelled albumin (Fig 3.8D). When CD4+ T cells were purified from OVA in alum immunized mice, IL-4 production was below detection levels for WT and Ship-/- cells.

3.3 Discussion

SHIP has been demonstrated to be a master negative regulator of hematopoietic cell survival, proliferation, differentiation and activation via the hydrolysis of the 5′-phosphate from the PI3K-generated product, PIP3 (Krystal, 2000). In this study, we sought to understand the role of SHIP in the regulation of DC generation, maturation and function. Comparing BM progenitors from Ship-/- mice with their WT littermate controls, we showed that SHIP negatively regulated DC generation but positively mediated DC maturation and activation in vitro and in vivo. Ship deletion results in more DCs being produced from BM precursors when cultured with GM-CSF, likely because of a higher sensitivity of Ship-/- progenitors to this cytokine. This hyper-responsiveness was also illustrated by enhanced survival of BM-derived Ship-/- DCs in the presence and absence of GM-CSF. These results differ from a previous report which found no difference in the number of DCs generated from WT and Ship-/- BM using GM-CSF in vitro (Neill et al., 2007). Possible explanations for this discrepancy could be the difference in mouse background: our studies were performed using mice backcrossed to C57Bl/6 at least 12 generations, while the previous study used F2 or F3 on a mixed 129/Sv and C57Bl/6J background. Additionally, differences in the culture methods used to generate the BM-derived DCs, specifically the addition of IL-4 and the absence of media changes in the previous study, could account for the differences. Together with enhanced survival and GM-CSF responsiveness, we found that Ship-/- DCs displayed
impaired maturation prior to and following TLR stimulation and produced less IL-12. This immature phenotype is likely responsible for the profound impairment of Ag-specific T cell proliferation induced by Ship-/- DCs as well as the reduced priming of a Th1 response.

SHIP has been shown to be tyrosine phosphorylated following the stimulation of many different receptors on hematopoietic cells (Huber et al., 1999; Krystal, 2000; Rohrschneider et al., 2000; Sly et al., 2007) and several studies have illustrated SHIP's role in myeloid cells, including macrophages (An et al., 2005; Rauh et al., 2005; Sly et al., 2009; Sly et al., 2004), mast cells (Damen et al., 2001; Kalesnikoff et al., 2002; Kalesnikoff et al., 2002), neutrophils (Strassheim et al., 2005) and myeloid derived suppressor cells (Ghansah et al., 2004; Paraiso et al., 2007). However, only one study has been published on the role of SHIP in DCs (Neill et al., 2007). Since SHIP-deficient mice have increased numbers of myeloid progenitors (Helgason et al., 1998), we determined the effect of SHIP on DC generation via CFSE dilution and demonstrated a more rapid generation of Ship-/- DCs from BM via enhanced dilution of CFSE in CD11c+ cells as well as faster generation of CD11c+ cells (Fig 3.2B and C). This enhanced generation of GM-DCs is recapitulated in vivo, where both a higher frequency and total number of CD11c+ cells were present in the spleen of SHIP-deficient mice. Interestingly, however, this enhancement was more remarkable in the CD8a+ population (Fig 3.3), not the myeloid CD8α- or CD11b+ populations as might be expected since such a finding would be in line with the myeloid hyperplasia of SHIP-deficient mice. Splenic Ship-/- DCs did, however, have lower levels of MHCII expression compared to WT DCs (Fig 3.3) which is consistent with the results of the GM-CSF-derived DC cultures (Fig 3.4). The hypersensitive response of Ship-/- BM progenitors to GM-CSF is likely due to enhanced activation of the PI3K pathway. Class IA heterodimeric PI3Ks function in receptor-mediated signalling pathways in the immune system, generating PIP3, which recruits proteins containing plekstrin homology (PH) domains to the plasma membrane (Koyasu, 2003a; Vanhaesebroeck et al., 2001a). In particular, Akt is recruited and positively regulates cell growth, survival and cytokine production. In the absence of SHIP, the threshold for activation of this pathway is reduced thus enhancing cell growth.
and survival (March and Ravichandran, 2002). A similar phenomenon has been documented in macrophages derived from SHIP-deficient mice (Rauh et al., 2003). Another protein that has been implicated, along with SHIP, in the negative regulation of macrophages is Lyn (Baran et al., 2003). Lyn-deficient mice, like SHIP-deficient mice, have perturbed myelopoiesis (Harder et al., 2004). Furthermore, Lyn is one of the Src-family members implicated in the tyrosine phosphorylation (and thus, perhaps plasma membrane recruitment) of SHIP (Baran et al., 2003; Hernandez-Hansen et al., 2004). The close relationship of Lyn and SHIP, and the similarities of their deleted phenotypes suggest the possibility that part of the phenotype of Lyn-deficient mice may be due to reduced activation of SHIP. Consistent with these findings, Lyn-/- DCs are also hypersensitive to GM-CSF, display an immature phenotype and produce less IL-12 upon TLR stimulation (Chu and Lowell, 2005). Given that SHIP is a substrate for Lyn, these similarities are not surprising and Lyn and SHIP probably lie on the same signalling pathway in DCs and other immune blood cells.

DCs are sentinels of the immune system. After encounter with a pathogen, innate immune activation results in DC maturation and development of highly efficient Ag presentation to T cells and subsequent initiation of adaptive immune responses (Villadangos and Schnorrer, 2007). Our data illustrate a role for SHIP as a positive regulator of DC maturation (Fig 3.4). Upon TLR stimulation Ship-/- DCs fail to mature appropriately and IL-12 production in response to a variety of TLR ligands is reduced in Ship-/- DCs (Fig 3.6). Although GM-DCs were initially thought to only express TLR4 and TLR9 (Iwasaki and Medzhitov, 2004), recent studies have shown that these cells are responsive to ligands that bind TLR1-2, TLR2-6, TLR3, TLR4, TLR5 and TLR9 (Alexopoulou et al., 2001; Dearman et al., 2008; Didierlaurent et al., 2004). Here we show mRNA expression of TLR1-4 and 6-9 (Fig 3.5), although in the absence of significant mRNA detected for TLR5, we still see responses generated via flagellin (Fig 3.6 and 3.7). This could suggest that TLR5 protein is long lived and generated at an earlier stage than the DCs we isolated for our analyses of TLR mRNA.
The principle function of DCs is to activate T cells and their ability to do this is closely linked to their maturation status. We have shown that *Ship*-/- DCs induce little if any proliferation of Ag-specific T cells compared to WT DCs (Fig 3.7). This functional result is consistent with their immature phenotype, which also explains their near complete inability to generate a T cell response (ie, only a very small percentage of cells with upregulated MHCII molecules and lower levels of other costimulatory molecules (Fig 3.4)). The immature phenotype of *Ship*-/- DCs likely couples with the reduced IL-12 production (in response to TLR-stimulation) leading to impaired priming of an effective T\(_{\text{H}1}\) response. Although IL-12 production was impaired from TLR-stimulated *Ship*-/- DCs, the production of IL-6 and TNF\(\alpha\) was not (Fig 3.6). Whether PI3K is a positive or negative regulator of TLR-induced cytokine production is a matter of much debate, with some claiming a positive role (Hattori et al., 2003; Hirsch et al., 2000; Li et al., 2003; Madrid et al., 2001; Weinstein et al., 2000a) and others suggesting a negative role (Aksoy et al., 2005; Diaz-Guerra et al., 1999; Guha and Mackman, 2002; Hazeki et al., 2006; Park et al., 1997; Tsukamoto et al., 2008) reviewed in (Hazeki et al., 2007). The controversy likely arises from the use of different cell types, assay kinetics and dependence on pharmacological inhibitors. However, the most recent studies favour a negative regulatory role for PI3K. Studies performed by one group has shown that PI3K is a negative regulator of IL-12 production by DCs induced by TLR stimulation and, in addition, mice deficient for the p85\(\alpha\) subunit of PI3K show enhanced T\(_{\text{H}1}\) responses (Fukao et al., 2002; Fukao and Koyasu, 2003). Although similar results have been obtained by several groups (Goodridge et al., 2003; Kuo et al., 2006; Martin et al., 2003) the proposed molecular mechanisms remain controversial. Interestingly, a study using *Giardia lamblia*, a flagellated protozoan, found that incubation of DCs with this parasite reduced TLR-induced IL-12 production and that PI3K activity was necessary for this reduction (Kamda and Singer, 2009). Further confirming these results, a study using PTEN-deficient DCs revealed they produced lower amounts of IL-12 than WT DCs following LPS stimulation (Ohtani et al., 2008). This finding is similar to our results using *Ship*-/- DCs. However, unlike the study using *Pten*-/- DCs (Ohtani et al., 2008), *Ship*-/- DCs, in general, also produce lower levels of IL-10 in response to TLR activation (Fig 3.6). Taken together, these results suggest that the second messenger PI-3,4-P\(_2\)
(generated by SHIP) may play a role in the pathways responsible for IL-10 production. As well, enhanced IL-10 production has been shown to contribute to reducing IL-12 levels (Martin et al., 2003), suggesting that other mechanisms may be responsible for the lower levels of IL-12 produced by Ship-/− DCs.

The reduced IL-12 production by Ship-/− DCs likely contributes to the impaired Th1 polarization observed when OVA peptide-loaded and TLR-stimulated Ship-/− DCs are cultured either in vitro or in vivo with OTII transgenic T cells (Fig 3.8A and B). This finding suggests that SHIP-deficient mice would show defects in Th1-type responses. This was indeed the case, as splenocytes and lymph node cells from SHIP-deficient mice immunized with OVA in CFA showed greatly reduced IFNγ secretion (a Th1 marker) upon re-stimulation with OVA (Fig 3.8C). This result was also confirmed when CD4+ T cells were purified from spleens and lymph nodes and co-cultured with WT or Ship-/− DCs, thus avoiding the problem of different proportions of B and T cells as well as the contributions of other cell types to T cell responses of whole splenocyte or lymph node cultures. Under conditions designed to skew towards a Th2 response (OVA in alum) SHIP-deficient mice were found to have enhanced responses as measured by IL-4 production from splenocytes (Fig 3.8C). These results match previous studies from our group showing that peritoneal macrophages from SHIP-deficient mice are M2 skewed, or alternatively activated (Rauh et al., 2005) – perhaps a consequence of enhanced IL-4 production by Ship-/− CD4+ T cells. In addition, SHIP-deficient mice have also been shown to have enhanced tumour growth (Rauh et al., 2005), indicative of a defect in Th1 activation and programming. Furthermore, SHIP-deficient mice suffer from a chronic asthmatic disorder, accompanied by infiltration of macrophages, lymphocytes, neutrophils and eosinophils into their lungs and enhanced production of the Th2 cytokines, IL-4 and IL-13. This suggests that SHIP negatively regulates Th2 signalling pathways (Oh et al., 2007). Although we have shown that the lung pathology in SHIP-deficient mice is likely due primarily to increased mast cell numbers and their enhanced responsiveness (Haddon et al., submitted 2009), DCs may also play a part.
Collectively these results illustrate the complexity of studying DC function and phenotype using SHIP-deficient mice, because of the loss of SHIP in other cell types. Indeed, the environment in which DCs develop plays an important role in determining the phenotype they acquired, as was illustrated by swap and transfer experiments with WT and *Ship*-/− DCs (Neill et al., 2007). As well, expansion of other cell types, in particular myeloid-derived suppressor cells may act to suppress T_{H}1 responses *in vivo* (Ghansah et al., 2004; Paraiso et al., 2007). Thus, as they become available, it will be very interesting to assess the DC phenotype and T_{H}1/ T_{H}2 proclivity of DC-lineage specific *Ship* knockout mice and to test the function of *Ship*-/− DCs *in vitro* and *in vivo* in the absence of the influence of a pan-hematopoietic *Ship*-/− milieu.

In summary, we have provided evidence that SHIP plays both a negative and positive role in GM-CSF-derived DC generation and maturation, respectively. Since dendritic cells modulate the nature and intensity of the adaptive immune response, DCs provide an attractive target for cancer therapy as well as control of autoimmune diseases. Recent advances in the development of small molecule activators of SHIP (Ong et al., 2007) have provided evidence that activation of SHIP could be used as an alternative to PI3K inhibition with the attractive quality that SHIP is hematopoietic-restricted while PI3K is more ubiquitous. Such pharmacological modulators of SHIP activity could be used to modify DC activity *in vitro* and *in vivo* and may provide a means of exploring SHIP activity in DC-based cell therapies.
CHAPTER 4: SHIP NEGATIVELY REGULATES Flt3L-DERIVED DENDRITIC CELL GENERATION AND POSITIVELY REGULATES MYD88-INDEPENDENT TLR-INDUCED ACTIVATION

4.1 Introduction

Dendritic cells (DCs), the most potent Ag-presenting cells, play critical roles in the induction of appropriate adaptive immune responses (Heath et al., 2004). DCs have been categorized into several subsets with unique phenotypes and functions. DCs are broadly classified into conventional DCs (cDCs, or myeloid DCs, expressing CD11c and CD11b) and a CD11c^{+}B220^{+} subset referred to as plasmacytoid DCs (pDC) (Shortman and Naik, 2007). The generation of DCs in the presence of GM-CSF, resulting in the production of cDCs, has long been the standard for in vitro DC studies (Inaba et al., 1992; Lutz et al., 1999). Recently, however, evidence suggests that the DCs derived in this manner possess more of an inflammatory phenotype rather than a steady state DC phenotype (Xu et al., 2007). In 2000, a novel method of DC derivation from BM was developed using the ligand for Flt3 (Flt3L or FL) (Brasel et al., 2000). Cells derived in the presence of Flt3L were found to express the classical DC marker CD11c, as well as CD86 and MHCII molecules, and could also be divided into two subsets based on expression of CD11b. In addition, these cells, much like GM-CSF-derived DCs (GM-DCs), could upregulate the maturation markers CD40, CD80, CD86, and, MHCII, in response to TLR activation. Further studies comparing the two culture systems found that Flt3L-derived DCs (FL-DCs) were morphologically and phenotypically distinct from GM-DCs (Weigel et al., 2002; Xu et al., 2007). In addition, Flt3L cultures were found to actually contain three DC subtypes including the pDCs (CD11c^{+}B220^{+}) and two subtypes of cDCs (CD11c^{hi}CD11b^{lo} and CD11c^{lo}CD11b^{hi}) (Brasel et al., 2000; Brawand et al., 2002; Gilliet et al., 2002). Plasmacytoid DCs represent approximately 30% of FL-DCs and are the main producers of type I IFNs (ie, IFNα and β) in response to TLR activation. Thus, Flt3L-derived BMDC cultures, as a whole, are more representative of steady-state splenic DCs (Naik et al., 2005).
In light of the known differences between GM-CSF- and Flt3L-derived DCs, as well as the different roles for PI3K in different cell types, we investigated the role of SHIP in the generation and innate activation of FL-DCs. The role of the PI3K pathway, or SHIP, in pDCs or in FL-DCs is not well characterized. One study using human pDCs demonstrated that inhibition of PI3K with LY294002 or wortmannin reduced the production of IFNα from TLR7- or TLR9-stimulated pDCs (Guiducci et al., 2008). In contrast, the production of IL-6 and TNFα and the expression of maturation markers were not affected by the addition of PI3K inhibitors to TLR7- or TLR9-stimulated pDCs. These results suggest that PI3K is a positive regulator of type I IFN production in human pDCs.

4.2 Results

4.2.1 Expression of negative regulators of the PI3K pathway in WT and Ship-/- Flt3L-derived DCs

The PI3K pathway is activated in response to multiple stimuli in immune cells and has a profound effect on the immune response of these cells. As such, it is critical that it be tightly regulated. By immunoblotting with specific antibodies, we found that SHIP-deficient DCs express similar levels of PTEN and SHIP2 to that present in WT DCs, indicating that loss of SHIP does not result in compensatory mechanisms to increase the negative regulation of PI3K by other lipid phosphatases in FL-DCs (Fig 4.1).

![Figure 4.1 Expression of negative regulators of the PI3K pathway in Flt3L-derived Ship-/- DCs.](image)

WT -/-

| SHIP2 |
| SHIP (P1C1) |
| PTEN |
| GAPDH |

Figure 4.1 Expression of negative regulators of the PI3K pathway in Flt3L-derived Ship-/- DCs. WT and Ship-/- DCs were harvested from day 8 cultures and the expression of SHIP, SHIP2 and PTEN were determined by immunoblotting. GAPDH was used as a loading control. The results are representative of three independent experiments.
4.2.2 Enhanced expansion of Flt3L-derived DCs from Ship-/- BM precursors in vitro

We showed in the previous chapter, that in the absence of SHIP, there is an enhanced generation of GM-DCs from BM progenitors. To determine if this was also true with Flt3L-derived cultures, freshly isolated BM cells from WT and Ship-/- mice were cultured with various concentrations of Flt3L. The non-adherent and loosely adherent cells from both cultures were, as the literature states, noticeably smaller and less granular than GM-CSF cultures (data not shown) and contained a comparable mix of the two broad DC subtypes (WT: CD11c⁺CD11b⁺ (cDCs) 54.04 ± 6.09%, CD11c⁺B220⁺ (pDCs) 24.86 ± 2.82%, KO: CD11c⁺CD11b⁺ (cDCs) 57.18 ± 5.3% CD11c⁺B220⁺ (pDCs) 30.5 ± 4.8%, n=5). We found that Ship-/- BM generated a higher frequency of Flt3L-derived CD11c⁺ cells, in keeping with a previous report (Neill et al., 2007). However, unlike this previous report, we found that the absolute numbers of total nucleated cells from Ship-/- FL-DC cultures were higher than from WT cultures and, when combined with the increased percentage of DCs, the Ship-/- BM cultures generated approximately three times the total number of DCs than WT BM cultures at all concentrations of Flt3L tested (Fig 4.2A, left panel). For WT and Ship-/- BM cultures, 100 ng/ml Flt3L yielded maximal DC numbers. Time course studies, using a dose of 100 ng/ml Flt3L, revealed that there were more total numbers of DCs (ie., CD11c⁺ cells) generated from Ship-/- than WT BM on each day of culture (Fig 4.2A, right panel). Like the GM-CSF cultures, we surmised that this difference was likely due to increased proliferation of Ship-/- DC precursors. However, as with GM-CSF-derived DCs, interpretation of the increased expansion of Ship-/- FL-DCs was complicated by the fact that Ship knock out mouse BM had approximately four fold higher numbers of DC progenitors than WT mouse BM (Neill et al., 2007). Moreover, as shown below, Ship-/- but not WT Flt3L-derived CD11c⁺ cells were capable of continued proliferation after they became CD11c⁺ cells. Therefore, we analyzed the generation of CD11c⁺ FL-DCs using CFSE to examine the proliferation of individual cells independent of precursor frequency. Five days after treatment with 125 mg/kg 5-fluorouracil (5-FU) to induce myeloablation, BM was isolated from Ship-/- and WT mice, labelled with 5 μM CFSE and cultured with Flt3L while we tracked CFSE dilution in the CD11c⁺ cell population. As shown in Fig 4.2B,
Figure 4.2: Enhanced expansion of Flt3L-derived DCs from Ship-/- BM precursors in vitro. CD11c+ DC numbers were enumerated using a hemocytometer and flow cytometry. (A) Left panel, BM cells isolated from WT and SHIP-deficient mice were cultured in 96 well plates containing 200 μl of nucleated cells at 1.5x10^6 c/ml. These BM cells were cultured with concentrations of Flt3L ranging from 0 to 500 ng/ml for 8 days. Absolute numbers of Ship+/+ (■) and -/- (Δ) CD11c+ cells on day 8 are shown as the mean ± SEM of triplicate determinations. Right panel, growth kinetics were determined by culturing BM cells with 100 ng/ml Flt3L in 12 well plates containing 1 ml of cells at 1.5x10^6 c/ml. Cells were harvested, total cells counted and analyzed by flow cytometry daily for 8 days. (B) BM cells isolated from 5-FU treated mice were labelled with CFSE and cultured with 100 ng/ml Flt3L. On the indicated day, CFSE dilution was determined in the CD11c+ population by flow cytometry. The mean florescence intensity (MFI) for the CD11c+ cells is shown. (C) Differentiation kinetics were determined daily during 8 days of culture with 20, 100 or 500 ng/ml Flt3L by measuring the percentage of CD11c+ cells. (D) Left panel, cells were harvested on day 8 of culture, washed thoroughly and cultured in pDC base medium with or without 10 ng/ml or 100ng/ml Flt3L. Cell survival was assessed by trypan blue exclusion. Right panel, survival kinetics in the presence of 100 ng/ml Flt3L and vehicle control (DMSO) or 5 μM of the PI3K inhibitor LY294002 (LY). All assays were performed in triplicate and results displayed are the mean ± SEM. Data in A, B, C and D are representative of two or three independent experiments. * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. Ship-/- DCs)
the generation of DCs in the presence of Flt3L was more rapid from Ship-/- than WT BM precursors. The Ship-/- BM cells showed not only increased cell division, but accelerated differentiation into CD11c+ cells (ie., the percentage of CD11c+ cells was higher in Ship-/- cultures). This accelerated differentiation was most apparent in the early cultures (days 3 and 4) and at low concentrations of Flt3L (Fig 4.2C). However, the percentage of CD11c+ cells in WT and Ship-/- cultures were equivalent by day 6.

Since the increased numbers of Ship-/- FL-DCs from whole BM cultures could also be the result of prolonged survival in response to growth factors, using trypan blue exclusion, we monitored the survival of day 8 differentiated WT and Ship-/- FL-DCs in the absence and presence (10ng/ml or 100ng/ml) of Flt3L (Fig 4.2D, left panel). Interestingly, unlike GM-DCs, Ship-/- FL-DCs survived only slightly better than WT DCs in the absence of Flt3L, where both Ship-/- and WT cells died rapidly. At 10 ng/ml Flt3L, the survival of Ship-/- DCs was significantly enhanced compared to WT. However, by the ninth day of culture, the majority of cells in both cultures were trypan positive (non-viable). At a higher concentration (100 ng/ml) of Flt3L (the same concentration used in their derivation), WT DCs were maintained without expansion while Ship-/- DCs showed considerable expansion up to day 5 followed by a steady decline in numbers. Enhanced PI3K activation is known to promote cell survival. Therefore, we tested if the expansion of Ship-/- DCs using 100 ng/ml Flt3L was the result of enhanced activation of the PI3K pathway using the PI3K inhibitor LY294002 (LY). Addition of LY to WT and Ship-/- cultures decreased cell survival, with a greater effect observed with the Ship-/- DCs (Fig 4.2D, right panel). These results are consistent with the notion that SHIP represses FL-DC survival by inhibiting the PI3K pathway.

4.2.3 Enhanced Akt activation in Flt3L-derived Ship-/- DCs following TLR activation

DCs act as sentinels of the immune system, detecting the presence of invading micro-organisms. Upon encountering such pathogens, stimulation of TLRs induces signalling events that culminate in the activation of transcription factors needed for
cytokine production and upregulation of co-stimulatory molecules (Beutler, 2009). To determine if SHIP regulates signalling downstream of TLR activation, we stimulated WT and Ship-/- DCs with 100 ng/ml LPS for the times indicated (Fig 4.3A). As shown in the top panel, we found that SHIP became tyrosine phosphorylated in WT DCs in response to

**Figure 4.3 Flt3L-derived Ship-/- DCs display enhanced Akt phosphorylation in response to LPS.** (A) LPS-induced signalling was assessed in day 8 Flt3L-derived DCs by stimulating with 100 ng/ml LPS for the indicated times, followed by SDS-PAGE. Immunoblot analysis was performed using phosphospecific antibodies and GAPDH was used as a loading control. (B) The expression of TLR4 and CD14 by the CD11c+ population was determined for day 8 cultures (black line). The grey area indicates the fluorescence of an isotype control antibody. The MFI of the CD11c+ cells is indicated.
LPS stimulation. We also found, as shown in the second and third panels, that STAT1 (S727) and Erk1/2 were phosphorylated to roughly the same extent in LPS-stimulated WT and Ship-/- DCs. However, Akt, the serine/threonine kinase downstream of PI3K, was substantially more phosphorylated in Ship-/- DCs. This difference in signalling was likely due to enhanced PI3K pathway activation in the absence of SHIP rather than a difference in cell surface expression of the LPS receptor since both TLR4 and CD14 levels were found to be comparable in WT and Ship-/- FL-DCs (Fig 4.3B).

4.2.4 Impaired maturation in Flt3L-derived Ship-/- DCs after innate immune activation

Since the development of an appropriate DC response is dependent on maturation induced by exposure to Ags and innate immune stimuli, we examined the ability of Ship-/- FL-DCs to mature following stimulation by TLR ligands. After 24 hrs in the presence or absence of 100 ng/ml LPS, 0.3 μM CpG or 50 μg/ml dsRNA, Ship-/- FL-DCs were less mature, as indicated by fewer cells expressing MHCII$^{\text{high}}$ or the co-stimulatory molecules, CD40, CD80 and CD86 (Fig 4.4A). Although reduced maturation occurred in response to all three TLR ligands, significant differences were only detected following LPS or dsRNA stimulus (Fig 4.4B). In addition, Ship-/- DCs expressed lower levels of most co-stimulatory molecules and MHCII in response to LPS and dsRNA (Table 4.1).
Figure 4.4 Flt3L-derived Ship-/- DCs fail to mature appropriately following TLR activation. (A) Day 8 DC cultures were stimulated with LPS, CpG and dsRNA for 24 hrs. The maturation of CD11c+ cells in media-treated (blue line) and TLR ligand-treated (red line) cultures was determined by assessing the expression of MHCII, CD40, CD80 and CD86 using flow cytometry (isotype control, grey line). The increase in percent positive of stimulated cells is indicated by the regional markers. Data shown are representative of three independent experiments. (B) The flow data obtained in (A) are expressed as percent WT (black bars) and Ship-/- (white bars) cells displaying CD40^{high}, CD80^{high}, CD86^{high} and MHCII^{high}. Data shown are the mean ± SEM * p<0.05; ** p<0.01 (WT vs. Ship-/- DCs)
A  

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- CD40  
- CD80  
- CD86  
- MHCII
Table 4.1 MFI of MHCII and costimulatory molecules for unstimulated and TLR-stimulated WT and Ship/-/- FL-DCs

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4.2.5 TLR activation-induced cytokine secretion is altered in Flt3L-derived Ship/-/- DCs

In addition to up-regulating co-stimulatory molecules on DCs, innate activation also plays an important role in shaping the adaptive immune response through the production of cytokines such as the pro-inflammatory cytokines, IL-12, IL-6 and TNFα and the anti-inflammatory cytokine, IL-10. We measured the secretion of these cytokines after DC stimulation with a variety of TLR ligands. The differences in cytokine production varied between WT and Ship/-/- FL-DCs when stimulated with different TLR ligands. At high doses of LPS, dsRNA and PGN, Ship/-/- DCs produced larger amounts of IL-12, while high doses of flagellin resulted in reduced IL-12 production. On the other hand, following CpG stimulation, we did not detect a difference in IL-12 production by WT and Ship/-/- DCs (Fig 4.5A, top panels). The production of IL-6 from WT and Ship/-/- DCs was similar in response to all TLR ligands with the exception of high doses of LPS, where significantly higher levels of IL-6 were secreted by Ship/-/- DCs (Fig 4.5A, second row of panels). TNFα secretion was higher by Ship/-/- than WT DCs over a range of doses in response to LPS, dsRNA, flagellin and PGN (although the increase was not statistically significant for PGN due to variable absolute amounts produced from experiment to experiment). However, no differences in TNFα secretion were observed between WT and Ship/-/- DCs when stimulated with CpG (Fig 4.5A, third row of panels).
Figure 4.5 Ship-/− DCs display altered cytokine production after TLR activation. DCs were isolated from day 8 cultures and treated with the indicated concentrations of LPS, CpG, dsRNA, flagellin and PGN for 24hrs at 37°C. Supernatants were assayed for IL-12, IL-6, TNFα and IL-10 by ELISA. The results shown are the mean ± SEM of three or four independent experiments performed in duplicate. * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. Ship-/− DCs) (B) Total RNA was isolated from day 8 WT and Ship-/− DCs and the expression of TLR transcripts determined by RT-PCR using 100 ng of template. Water (H₂O) without template was used as a negative control. Data shown are representative of three independent experiments.
IL-10 (pg/ml)  TNFα (pg/ml)  IL-6 (pg/ml)  IL-12 (pg/ml)

LPS (ng/ml)  CpG (μM)  dsRNA (μg/ml)

*  **  ***  ****

+/+  +/-
B

IL-12 (pg/ml)

IL-6 (pg/ml)

TNFα (pg/ml)

IL-10 (pg/ml)

flagellin (ng/ml) PGN (μg/ml)

TLR1 TLR2 TLR3 TLR4 TLR5 TLR6 TLR7 TLR8 TLR9

100 bp ladder

+/- +/+-/- +/+-/- +/+-/-+/+-/- +/+-/-+/+-/- +/+-/- +/+-/- H2O
Lastly, IL-10 levels were significantly higher in *Ship-/-* DCs in response to LPS and CpG stimulation, while lower in response to dsRNA and flagellin and not different in response to PGN (Fig 4.5A, bottom panels). Of note, the level of IL-10 produced by dsRNA and flagellin stimulation was very low compared to that stimulated by the other TLR agonists. To summarize, LPS stimulation resulted in enhanced production of IL-12, IL-6, TNFα and IL-10 from *Ship-/-* FL-DCs, with the greatest difference observed using high doses of LPS. CpG stimulation resulted only in enhanced IL-10 production by *Ship-/-* DCs and no differences in the other cytokines analyzed. dsRNA stimulation caused *Ship-/-* DCs to produce more IL-12 and TNFα but comparable levels of IL-6 and lower levels of IL-10. Flagellin stimulation also resulted in enhanced TNFα levels from *Ship-/-* DCs, but lower IL-12 levels. Lastly, with PGN stimulation only IL-12 production by *Ship-/-* DCs was significantly increased over WT controls.

Differences in these responses were likely not the result of altered TLR expression since, in addition to equivalent cell surface expressed TLR4, WT and *Ship-/-* DCs were found to have comparable levels of TLRs 1, 2, 6, 8 and 9 transcripts (Fig 4.5B). Although little or no TLR3 mRNA was detected in WT or *Ship-/-* FL-DCs, we observed significant stimulation of cytokine production with dsRNA—this may be because dsRNA can also act through RIG1 and MAVs, providing an alternate MyD88-independent pathway of activation (Beutler et al., 2007). Similarly, TLR5 was not detected by RT-PCR, but flagellin still stimulated cytokine production. It is possible that TLR5 mRNA is transcribed only during differentiation and the resulting protein is long lived on mature FL-DCs.

### 4.2.6 Enhanced cytokine production in *Ship-/-* DCs in response to intact bacteria

*In vivo* activation of DCs normally occurs through the stimulation of multiple TLRs by intact pathogens. Thus, we stimulated WT and *Ship-/-* DCs with intact *Caulobacter crescentus*, a Gram negative flagellated bacterium which dies over 1 to 2 days at 37°C, releasing intracellular components as well as causing activation through cell wall components. Thus, *Caulobacter crescentus* treatment stimulates multiple TLRs
Figure 4.6: Flt3L-derived Ship-/- DCs have enhanced responses to whole bacteria. Day 8 DCs were stimulated with Caulobacter crescentus at the indicated ratio for 24 hrs. Supernatants were collected for cytokine analysis by ELISA. Results shown are the mean ± SEM from three independent experiments performed in duplicate. * p<0.05; ** p<0.01 (WT vs. Ship-/- DCs)

at the cell surface and in endosomes. We found that Ship-/- DCs secreted substantially higher levels of TNFα and IL-10, but not IL-12 or IL-6, when stimulated with various doses of Caulobacter crescentus (Fig 4.6).

4.2.7 SHIP regulates TLR-induced IFN-β production

An important function of pDCs is the production of high levels of type I IFN in response to viral infections. Therefore, we examined the production of IFNβ in response to TLR stimulation. According to the literature, stimulation of TLR3 and 4 induce IFNβ production through MyD88-independent pathways (Kawai et al., 2001; Yamamoto et al., 2003a) whereas TLR9 stimulation produces IFNβ through a MyD88-dependent pathway
(Honda et al., 2005). As shown in Figure 4.7, LPS induced very low levels of IFNβ from WT and Ship-/- DCs while both CpG and dsRNA stimulation resulted in robust IFNβ secretion. However, SHIP’s role in the regulation of IFNβ production downstream of CpG and dsRNA stimulation was quite different. Specifically, stimulation of Ship-/- FL-DCs with CpG resulted in reduced IFNβ production, whereas dsRNA stimulation caused increased IFNβ production compared to WT FL-DCs. These results suggest that SHIP positively regulates MyD88-dependent IFNβ production and negatively regulates MyD88-independent IFNβ production.

Figure 4.7 SHIP regulates TLR-induced IFNβ production. Day 8 DCs were stimulated with the indicated TLR ligand for 24hrs (LPS, 100 ng/ml; CpG, 0.3 μM; dsRNA 50 μg/ml). Supernatants were collected for IFNβ ELISA. Results are expressed as the mean ± SEM of three independent experiments performed in duplicate. * p<0.05; ** p<0.01 (WT vs. Ship-/- DCs)

4.2.8 Ship-/- Flt3L-derived DCs induced by MyD88-independent TLR signalling pathways display impaired activation of Ag-specific T cell proliferation in vitro

The primary function of activated DCs is to induce Ag-specific T cell proliferation and activation resulting from multiple signals stemming from a combination of Ag presentation, co-stimulatory surface molecules and cytokine production. To assess the relative ability of WT and Ship-/- FL-DCs to stimulate T cells we used an in vitro Ag-
specific T cell proliferation assay. CD4+ T cells isolated from OTII transgenic mice were co-cultured with WT or Ship-/- DCs loaded with OVA_{323-339} peptide and activated with a TLR ligand for 72 hrs after which we measured T cell proliferation by ^3_H-thymidine incorporation. Using this method, we found that Ag-specific induction of T cell proliferation by DCs was greatly reduced when Ship-/- DCs were stimulated with LPS or dsRNA (ligands that trigger MyD88-independent pathways) but no differences were detected when WT and Ship-/- DCs were activated with CpG, flagellin or PGN, which trigger only MyD88-dependent pathways (Fig 4.8). The impaired ability of LPS- and dsRNA-stimulated Ship-/- FL-DCs to promote T cell proliferation correlated with reduced maturation of Ship-/- DCs in response to these two TLR ligands (Fig 4.4B).

**Figure 4.8: Ship-/- Flt3L-derived DCs have a reduced ability to induce Ag-specific T cell proliferation.** CD4+ T cells were isolated from OTII transgenic mice and cultured with TLR-activated DCs and the indicated concentration of OVA_{323-339}. T cell proliferation was determined by ^3_H-thymidine incorporation. Results displayed are the mean ± SEM of triplicate determinations. Results are representative of at least three independent experiments.
4.2.9 *Ship*-/- Flt3L-derived DCs promote TH1 responses *in vitro*

DCs play a pivotal role in determining the TH1/TH2 characteristics of an immune response. To determine if the aberrant phenotype and function of *Ship*-/- DCs affected the character of the immune responses they generated, we compared WT and *Ship*-/- DC-induced TH responses *in vitro* and *in vivo*. CD4\(^+\) OTII T cells were activated *in vitro* by OVA peptide-loaded DCs following treatment with TLR ligands. Co-culture supernatants were collected and assayed for IFN\(\gamma\) and IL-4. We found that Ag-specific IFN\(\gamma\) production was significantly higher in T cells co-cultured with TLR ligand-treated *Ship*-/- DCs (except when CpG was used) (Fig 4.9A). IL-4 production was not detectable in any of the cultures. To study T cell polarization *in vivo*, we intravenously injected OVA peptide-loaded and LPS-activated WT or *Ship*-/- FL-DCs into OTII transgenic mice. The TH responses were measured four days later by culturing total splenocytes isolated from DC-immunized mice with OVA peptide or by isolating splenic CD4\(^+\) T cells and co-culturing them with LPS-activated WT or *Ship*-/- DCs loaded with OVA peptide. In contrast to our *in vitro* results, the production of IFN\(\gamma\) was not significantly different from either the whole splenocytes or the CD4\(^+\) T cells (Fig 4.9B). The production of IL-4 was only detectable in splenocyte cultures, with *Ship*-/- immunized OTII mouse splenocytes producing similar levels of IL-4 to WT DC-immunized OTII mouse splenocytes (WT = 55.8 ± 2.8 pg/ml; *Ship*-/- = 53.9 ± 5.2 pg/ml). In a parallel experiment to that shown in Chapter 3 (Fig 3.8C), CD4\(^+\) LN cells and splenocytes purified from mice that were immunized with OVA in CFA were co-cultured with OVA-loaded FL-DCs. In the absence of TLR stimulation, naïve *Ship*-/- FL-DCs had a reduced ability to induce TH1 responses (based on IFN\(\gamma\) production) in primed splenic or LN CD4\(^+\) T cells from OVA/CFA immunized *Ship*-/- mice (Fig 4.9C). When the same cells were cultured with CD4\(^+\) cells from OVA/alum immunized mice, IL-4 production was not detectable. The differences observed were not the result of aberrant OVA capture, since WT and *Ship*-/- DCs had similar abilities to uptake FITC-labelled albumin (Fig 4.9D).
Figure 4.9: *Ship*-/- Flt3L-derived DCs induce altered Th1 responses *in vitro* but not *in vivo*. (A) CD4+ OTII T cells were co-cultured with OVA<sub>323-339</sub> peptide-loaded TLR-activated WT or *Ship*-/- DCs and supernatants collected after 4 days for assessment of IFNγ by ELISA. (B) OVA<sub>323-339</sub> peptide-loaded LPS-activated WT or *Ship*-/- DCs were injected (iv) into OTII transgenic mice. Splenocytes were collected 4 days later and cultured with 1 μg/ml OVA<sub>323-339</sub> peptide, or CD4+ T cells were purified and cultured with LPS-stimulated OVA<sub>323-339</sub> peptide-loaded WT or *Ship*-/- DCs (CD4/BMDC). Supernatants were collected after 4 days of co-culture and subjected to IFNγ ELISA. (C) WT or SHIP-deficient mice were immunized by injection (sc) of OVA in CFA. After 10 days, CD4+ cells from LNs and spleen were purified and co-cultured with OVA -loaded WT and *Ship*-/- Flt3L-derived DCs (CD4/BMDC). Supernatants were collected after 3 days of co-culture and subjected to IFNγ ELISA. For Figs A, B and C, results shown are the mean ± SEM from three independent experiments performed in duplicate. (D) WT and *Ship*-/- DCs were incubated with FITC-albumin and its uptake at 4°C (■) and 37°C (black line) monitored by flow cytometry. * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. *Ship*-/- DCs)
4.3 Discussion

In this study we demonstrate that SHIP plays a role in the generation and innate activation of FL-DCs. Although few studies have been conducted using FL-DCs, it has been shown that their phenotype and functions differ significantly from those derived under the standard culture conditions of GM-CSF±IL-4 (Xu et al., 2007). In this study, we have shown that SHIP plays a common role in the generation of DCs in both Flt3L and GM-CSF culture systems but differs in its effects on DC activation and function.

Specifically, when we cultured BM in Flt3L we found that SHIP negatively regulated the generation of these DCs, ie., Ship-/- BM precursors produced more DCs than WT BM precursors at all doses of Flt3L tested. In addition, Ship-/- BM yielded CD11c+ cells faster at the standard culture concentration of 100 ng/ml Flt3L (Fig 4.2A), most likely because of enhanced sensitivity of SHIP-deficient DC precursors to this cytokine. In addition, we demonstrated a more rapid generation of Ship-/- FL-DCs by CFSE dilution and CD11c+ differentiation (Fig 4.2B and C) – showing enhanced proliferation in addition to more rapid generation. Survival assays with DCs that had been derived with Flt3L for 8 days, revealed that, in the absence of Flt3L, Ship-/- DCs have slight survival advantage over WT DCs - this advantage is more pronounced at sub-optimal concentrations of Flt3L (Fig 4.2D). Interestingly, we found that upon re-culturing with a culture concentration of Flt3L (100 ng/ml), differentiated Ship-/- DCs continue to expand whereas WT DCs merely survive without expansion. Related to this, the PI3K pathway has long been known to enhance cell growth and survival (Deane and Fruman, 2004; Weichhart and Saemann, 2008). We therefore wanted to determine if enhanced PI3K pathway activation, as a result of SHIP-deficiency, might be the cause of the increased expansion observed in Ship-/- FL-DCs. To test this, we added the PI3K inhibitor, LY294002, and found, as expected, that it reduced the survival of WT FL-DCs and prevented the expansion of Ship-/- DCs. This demonstrates that SHIP plays a similar role in Flt3L- and GM-CSF-derived DCs, ie., restraining proliferation, differentiation and survival. SHIP likely exerts these effects by attenuating the PI3K pathway.
The PI3K pathway has been implicated in signalling downstream of TLRs (Hazeki et al., 2007; Weichhart and Saemann, 2008). Stimulation of TLRs activates adaptor molecules at the membrane and this leads to recruitment of class I PI3K to the receptor complex (Fruman, 2004). After receptor engagement, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PI-4,5-P$_2$) to generate PIP$_3$, a second messenger which recruits PH domain containing proteins, including Akt, to the intracellular surface of the plasma membrane. The production of PIP$_3$ can be negatively regulated by the lipid phosphatase PTEN that, when activated, removes the 3'$\text{p}$ phosphate group from PIP$_3$, regenerating PI-4,5-P$_2$ (Krystal, 2000). In addition, activated SHIP hydrolyzes the 5'$\text{p}$ phosphate group, generating another second messenger, PI-3,4-P$_2$. We investigated whether there were any signalling anomalies in Ship$^{-/-}$ FL-DCs following LPS stimulation and demonstrated, for the first time, that SHIP is phosphorylated in FL-DCs in response to LPS (Fig 4.3A). LPS stimulation also induced Akt activation, which was profoundly enhanced in Ship$^{-/-}$ FL-DCs, consistent with SHIP being a negative regulator of TLR-induced PI3K signalling in these cells. However, we did not detect significant differences in phosphorylation of STAT1 or Erk1/2.

Like GM-DCs, FL-DCs act as a bridge between the innate and adaptive immune system, generating comparable allogeneic T cell responses (Xu et al., 2007). A key component of DC-induced T cell activation is the ability to upregulate co-stimulatory and MHCII molecules (Colonna et al., 2004). Although pDCs are generally thought to express only TLRs 7 and 9 (Gilliet et al., 2008), since FL-DCs contain both pDCs and cDCs, they are capable, as a population, of responding to a variety of TLR ligands (Xu et al., 2007). Our results suggest that while SHIP is involved in FL-DC maturation following TLR stimulation, the difference between WT and Ship$^{-/-}$ DC maturation does not reach statistical significance with all TLR ligands (Fig 4.4B).

TLR signalling proceeds via either MyD88-dependent, MyD88-independent pathways, or, both. LPS binding to TLR4 occurs at the cell surface and activates both MyD88-dependent and -independent pathways. In terms of the MyD88 pathway, LPS-induced TLR4 dimerization recruits MyD88 (and TIRAP, aka MAL) via TIR-TIR
interactions and this stimulates IRAK4, IRAK1, TRAF6 and TAK1 to activate MAPK family members and the transcription factor NFκB, inducing downstream events such as cytokine production. TLR4 also activates a MyD88-independent pathway using a heterodimer of TRIF and TRAM to produce type I IFNs and upregulate co-stimulatory molecules (Kawai and Akira, 2007; Trinchieri and Sher, 2007). Double stranded RNA, on the other hand, does not bind to receptors at the cell surface but to intracellular TLR3 in endosomes, which signals strictly through a MyD88-independent pathway or, alternatively, in the cytoplasm via the RNA helicases, retinoic acid-inducible gene1 (RIG1) and Mda5 (Beutler et al., 2007). Non-methylated CpG DNA also binds its receptor, TLR9, in endosomes but activates only MyD88-dependent pathways, similar to those employed by TLR4. In response to TLR ligands activating MyD88-independent pathways (LPS and dsRNA), we found that *Ship*-/- FL-DCs were less able to upregulate co-stimulatory and MHCII molecules (Fig 4.4A and B) while CpG stimulation, which only stimulated the MyD88 pathway, did result in a reduced percentage of CD40⁺, CD86⁺ and MHCII⁺ cells, but these differences were not statistically significant. This is consistent with the possibility that SHIP functions to positively regulate only MyD88-independent pathways in FL-DCs. These results are in contrast to previous findings by our group showing that, in macrophages, the induction of SHIP was found to be uniquely MyD88-dependent (Sly et al., 2009). This provides yet another example of the PI3K pathway playing different roles in response to different stimuli and in different cell types.

Another important step in DC-induced T cell polarization is the production of pro-inflammatory cytokines. Interestingly, while LPS uses the MyD88-dependent pathway to trigger pro-inflammatory cytokine production and the MyD88-independent pathway to upregulate co-stimulatory molecules (Kawai and Akira, 2007; Trinchieri and Sher, 2007), dsRNA works only through the MyD88-independent pathway while CpG uses only the MyD88-dependent pathway to upregulate both cytokines and co-stimulatory molecules. Thus, since we found that SHIP positively regulated maturation of FL-DCs when MyD88-independent pathways were stimulated (ie., with LPS and dsRNA), we predicted less pro-inflammatory cytokine production from dsRNA- or LPS-induced WT than from *Ship*-/- FL-DCs. We found that CpG, similar to its effects on co-stimulatory molecule
expression, showed no statistical differences in pro-inflammatory cytokine production between WT and Ship-/- FL-DCs. This is in agreement with a study using human pDCs in which PI3K inhibition did not affect IL-6 or TNFα secretion or co-stimulatory marker levels in response to CpG stimulation (Guiducci et al., 2008). However, in contrast to the production of pro-inflammatory cytokines, IL-10 production was enhanced in CpG- (and LPS-) stimulated Ship-/- DCs (Fig 4.5A). This is consistent with a study in macrophages which demonstrated a positive role for PI3K in LPS-induced IL-10 production (Polumuri et al., 2007). Unlike CpG, the result with dsRNA stimulation did not give the expected cytokine profiles - Ship-/- FL-DCs secreted significantly more pro-inflammatory IL-12 and TNFα (Fig 4.5A). A possible explanation for this finding is that different cell populations within the Flt3L cultures are responding to the different TLR ligands. Related to this, it has been reported that pDCs respond to CpG-containing DNA but not to viral dsRNA, while cDCs are capable of responding to dsRNA (Kadowaki et al., 2001). Therefore, the complementary response of the two DC subsets present in Flt3L cultures may be responsible for our results. However, since we did not find any differences in the subset distributions between WT and Ship-/- cultures, we must eliminate this explanation for the differences in responsiveness of the two genotypes. LPS stimulation followed a similar pattern to that of dsRNA, where Ship-/- DCs produced increased levels of pro-inflammatory IL-12, IL-6 and TNFα, again suggesting a unique role for SHIP in regulating MyD88-independent responses in FL-DCs. Taken together, these results illustrate functional differences in the role of SHIP in GM-CSF-derived and Flt3L-derived BMDCs. In GM-DCs, SHIP had a universally positive role in innate activation of DCs, while the function of SHIP in FL-DCs depends on the activating stimulus.

During pathogen infection/exposure in vivo, it is often the case that DCs will be stimulated by multiple TLR ligands simultaneously (Trinchieri and Sher, 2007). Therefore, we stimulated WT and Ship-/- FL-DCs, which are purported to best represent steady-state splenic DCs (Naik et al., 2005), with Caulobacter crescentus. Caulobacter crescentus, a gram negative bacterium, will stimulate DCs through TLR4 (LPS cell membrane), TLR5 (flagellum) and TLR9 (via bacterial non-methylated CpG motifs in its
DNA). Despite the multi-ligand activation, we did not detect enhanced cytokine production compared to stimulation with any individual TLR ligand in WT DCs. However, we did find that Ship-/- FL-DCs did produce enhanced levels of TNFα and IL-10, but not IL-12 or IL-6 (Fig 4.6) compared to WT FL-DCs. This cytokine profile is reminiscent of low doses of LPS, suggesting that TLR4 signalling is causing the major effects seen in response to Caulobacter crescentus and the simultaneous activation of other TLRs does not influence the profile.

A primary function of pDCs is the production of large amounts of Type I IFNs in response to viral infection (Colonna et al., 2004; Liu, 2005). We found that when FL-DCs were stimulated with the viral components, CpG and dsRNA, SHIP had both positive and negative effects on IFNβ production (Fig 4.7). IFNβ production downstream of TLR9 (CpG) is known to be MyD88-dependent and relies on the transcription factor interferon regulatory factor 7 (IRF7) (Honda et al., 2005) while TLR3 (dsRNA) production of IFNβ is MyD88-independent and uses IRF3 (Schafer et al., 1998; Taniguchi et al., 2001). Studies investigating the role of PI3K in IFNβ production have found that inhibition of PI3K enhances IFNβ production downstream of TLR3 and 4 (Aksoy et al., 2005) but prevents IFNα secretion in response to TLR9 activation (Cao et al., 2008; Guiducci et al., 2008). At odds to these previous reports, we found that enhanced PI3K activity, because of SHIP-deficiency, caused reduced IFNβ production in response to CpG and enhanced IFNβ in response to dsRNA stimulation (Fig 4.7). Since some of these opposing previous studies were conducted using human pDCs, splenic isolated pDCs or cell lines (and not FL-DCs) the different conclusions compared to our results are not surprising since PI3K is known to have different effects in different cell types. As was the case with pro-inflammatory cytokine production, the IFNβ produced by the different TLR agonists could come from the different DC subtypes, ie pDCs producing IFNβ in response to CpG and cDCs producing IFNβ in response to dsRNA. This cell type difference could contribute as well to the opposing effects of SHIP in response to the two TLR ligands with SHIP positively regulating MyD88-dependent IFNβ production in pDCs and negatively regulating MyD88-independent IFNβ.
production from cDCs. This presents the intriguingly possibility that SHIP-deficient mice may have an enhanced ability to clear a dsRNA virus infection but a reduced ability to clear a DNA virus.

Another major finding of this study was the failure of LPS- or dsRNA- activated Ship-/- FL-DCs to effectively activate Ag-specific T cell responses (Fig 4.8). Conversely, CpG-, flagellin- and PGN-stimulated Ship-/- DCs were fully competent in the activation of Ag-specific T cell proliferation. These results correlate with the reduced expression of co-stimulatory molecules and MHCII in response to these ligands on Ship-/- FL-DCs (Fig 4.4A and B). In addition, these disparate results provide further evidence of a predominantly MyD88-independent role for SHIP in TLR-induced DC maturation. Notably, the impaired T cell proliferation appeared more robust than the reduced maturation (by cell surface phenotype markers), in particular for dsRNA stimulated Ship-/- FL-DCs. This may be explained by signals that originate from the T cells and are transduced to the DCs. DC function is influenced by CD28 and CD40L expressed on the surface of T cells (O'Sullivan and Thomas, 2003a; O'Sullivan and Thomas, 2003b; Orabona et al., 2004). Therefore, signals emanating from the T cell through Ship-/- DCs may be impaired, further reducing the ability of Ship-/- DCs to activate T cells. Intriguingly, these results are in opposition to the IFNγ production from T cells in the same in vitro co-cultures (Fig 4.9A). Although the maturation in response to LPS and dsRNA was reduced, as mentioned, the pro-inflammatory cytokines produced by the Ship-/- FL-DCs were enhanced. Thus Ship-/- FL-DCs' enhanced cytokine production may induce TH1 skewing in the absence of strong proliferative responses.

Overall, this study provides evidence for SHIP as a dual regulator of Flt3L-derived DC differentiation and TLR-induced cytokine production and maturation. Importantly, positive regulation of maturation and subsequent T cell activation by SHIP was found to be strictly a MyD88-independent phenomenon. Our results add to the relatively few reported studies investigating the responsiveness of FL-DCs to innate activation. We have found very intriguing evidence that SHIP, and likely the PI3K pathway, has both positive and negative regulatory roles in the TLR-induced activation of
DCs derived in the presence of Flt3L. Further study will be required to determine the relative influence of the different DC subtypes contained within this mixed culture.
CHAPTER 5: SHIP-DEFICIENT MICE DEVELOP EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN THE ABSENCE OF ENHANCED TH RESPONSES

5.1 Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inducible mouse model of multiple sclerosis (MS) characterized by T cell-mediated auto-inflammatory disease of the central nervous system (CNS) leading to demyelination (Kuchroo et al., 2002). In mice susceptible to developing EAE, disease is induced by immunization with neural Ags such as proteolipid protein (PLP), myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) in complete Freund's adjuvant. EAE was originally considered a prototypical Th1-mediated autoimmune disease. However, IFNγ-deficient mice, rather than being protected from EAE, have enhanced disease (Ferber et al., 1996). Recent studies suggest that IL-17-producing Th cells (Th17) are in fact primarily responsible for EAE pathogenesis since IL-17-deficient mice show delayed onset of disease and reduced severity (Komiyama et al., 2006) and neutralizing IL-17 prevents development of EAE (Park et al., 2005). Following the entry of autoreactive Th1 and Th17 cells, which contribute to disease, Foxp3+ regulatory T cells (Tregs) enter sites of CNS inflammation where they secrete IL-10 and TGFβ and limit disease pathology (Korn et al., 2007b; McGeachy et al., 2005; O'Connor and Anderton, 2008; O'Connor et al., 2007). Thus, mice that have depleted Tregs develop more severe EAE, while mice supplemented with Tregs are protected (Kohm et al., 2002; Mekala and Geiger, 2005; Montero et al., 2004; Zhang et al., 2004). In fact, pertussis toxin, an EAE model adjuvant, long thought to facilitate disease by permeabilizing the blood-brain barrier, may act in part by reducing Treg numbers in the spleen (Chen et al., 2006a). Inflammatory monocytes (IMCs) are also induced during the EAE disease course and are thought to be involved in suppression of EAE symptoms. IMCs are CD45+ cells identified by the surface Ag phenotype CD11b+Ly6CshLy6G+ (Zhu et al., 2007). Splenic IMCs increase substantially with EAE induction and are capable of suppressing T cell proliferation and inducing T cell death in vitro through the production of nitric oxide (NO).
In addition to T cell mediated disease mechanisms, B cells have also been found to play a role in the development of EAE (Ziemssen and Ziemssen, 2005). Although auto-Abs are not required for induction or progression of EAE, MOG-specific Abs can enhance inflammation and demyelination (Linington et al., 1988a; Lyons et al., 1999). A recent study found that two subsets of B cells play opposing roles in the development of EAE. Specifically, regulatory B cells (CD1dhiCD5+) appear to suppress EAE induction, whereas other B cells are required for the expansion of auto-Ag-specific T cells for disease progression (Matsushita et al., 2008). Lastly, DCs orchestrate EAE immune responses via Ag presentation and cytokine production (Manuel et al., 2007).

The phenotype of SHIP-deficient mice offers a very interesting model in which to study the pathogenesis of EAE. The original characterization of SHIP-deficient mice showed that Ship−/− mice have a myeloproliferative disorder, resulting in increased numbers of granulocytes and macrophages and, in contrast, reduced B cell numbers (Helgason et al., 1998). Further characterization of specific cell types isolated or derived from SHIP-deficient mice revealed other abnormalities. For example, SHIP-deficient mice have enhanced serum immunoglobulin (Ig) levels (Helgason et al., 2000), lack marginal zone B cells (Brauweiler et al., 2000; Helgason et al., 2000; Karlsson et al., 2003), and, mature B cells that are present have an activated phenotype and enhanced sensitivity to BCR stimulation. Much of the B cell phenotype in Ship−/− mice has been attributed to the consequence of the SHIP-deficient environment rather than an intrinsic B cell defect (Karlsson et al., 2003). This microenvironment-mediated defect has also been invoked to explain why MΦs isolated from SHIP-deficient mice are M2 skewed, or alternatively activated, expressing high levels of arginase 1 and Ym1 (Rauh et al., 2005). Although the development of classic Th and TCTL thymocyte compartments does not appear to be affected by SHIP deletion, peripheral T cells from SHIP-deficient mice have been shown to have a constitutively active phenotype and generate increased numbers of CD4+CD25+ Tregs (Kashiwada et al., 2006). In a recent study, both germline and inducible SHIP deletion led to increased CD4+CD25+FoxP3+ and CD4+CD25+FoxP3+ Treg numbers and delayed allograft rejection, highlighting an immunosuppressive
environment in SHIP-deficient mice (Collazo et al., 2009). However, in a T cell-specific
SHIP-deficient model, no differences in the activation state or number of Tregs are
observed (Tarasenko et al., 2007). In fact, these mice fail to skew towards a \( T_{H2} \)
response (Tarasenko et al., 2007).

Taken together, a SHIP-deficient mouse model presents an interesting array of
microenvironment and cell-autonomous defects for the study of EAE development and
disease progression. The contrast of hyper-responsive B cells, increased regulatory T
cells, M2 M\( \Phi \)s, increased numbers and hyper-responsive myeloid cells (including
increased myeloid suppressor cells (Ghansah et al., 2004)) and immature DCs (Neill et
al., 2007) presents an immune environment of opposing forces. In the studies described
in this chapter we investigated the effect of SHIP-deficiency on the development of EAE.
Due to the severe phenotype and short lifespan of SHIP-deficient C57BL/6 congenic
mice, the mice used in this chapter were an F2 hybrid consisting of a mixed C57Bl/6 x
129Sv background.

5.2 Results

5.2.1 SHIP is expressed in CNS-resident microglia

Although not definitively established, the current consensus is that microglial
cells are BM-derived hematopoietic cells that migrate as monocytes to the brain and fully
differentiate into microglia (Chan et al., 2007). Thus, microglia are considered CNS
resident M\( \Phi \)s which, when activated, contribute to EAE pathogenesis. Because of the
contentious hematopoietic origins of microglia, we first asked if SHIP was expressed in
CNS resident microglia by intracellular FACS staining as well as by Western blot
analysis and found, as shown in Fig 5.1A, that it was. Since SHIP-deficient mice possess
an increased number of myeloid cells, in particular M\( \Phi \)s, we enumerated the microglia
content of both naïve and EAE-induced WT and SHIP-deficient mice. However, we
found no significant difference in the percent of microglia in WT and SHIP-deficient brains and spinal cords, both in naïve and EAE-induced mice (Fig 5.1B).

Figure 5.1 SHIP is expressed in microglia.  A) Left panel, spinal cords from WT and SHIP-deficient mice were processed and microglia (CD45\(^+\)CD11b\(^+\)) were analysed for expression of SHIP by intracellular staining (WT red line, KO blue line).  Data shown are representative of 3 separate mice. Right panel, microglia (CD45\(^+\)CD11b\(^+\)) were FACS sorted from pooled brains and spinal cords of WT and SHIP-deficient mice and subjected to Western blot analysis for SHIP expression.  GAPDH is used as a loading control.  Data shown are representative of two independent experiments.  B) Brains and spinal cords from naïve and EAE-induced mice were processed and stained for CD45 and CD11b and analysed for the percentage CD11b\(^+\) within the CD45\(^+\) population.  Data shown are the mean ± SEM of 2 naïve WT and SHIP-deficient mice and 5 EAE-induced WT and SHIP-deficient mice.
5.2.2 SHIP-deficient mice are susceptible to EAE

We examined the susceptibility of SHIP-deficient mice to EAE by immunizing mice with MOG$_{35-55}$ and monitoring the mice daily for clinical symptoms (Fig 5.2A). All WT mice developed typical EAE symptoms, starting approximately 10 days after immunization with MOG$_{35-55}$ (plus CFA) and pertussis toxin. All but two SHIP-deficient mice developed disease with 6 of these dying as a result, compared to 2 deaths in the WT group (Fig 5.2B and C). Although there appeared to be a delay in the onset of EAE in the SHIP-deficient mice, we were surprised that they developed it at all, given that EAE is considered either a prototypical T$_{H1}$-or T$_{H17}$-mediated autoimmune disease and, as shown in Chapter 3, SHIP-deficient DCs are very poor at presenting Ags and stimulating a T$_{H1}$ response (ie., stimulating IFN$_{\gamma}$ production). We therefore investigated the mechanism(s) by which SHIP-deficient mice developed EAE.
Figure 5.2 SHIP-deficient mice are susceptible to EAE.  

A) Time course of the clinical score of WT and SHIP-deficient mice immunized with MOG_{35-55} as described in Materials and Methods.  

B) Time course of the incidence ratio of WT and SHIP-deficient mice (calculated as the number of mice with disease per total number of induced mice).  

Data shown are the mean ± SEM (WT, n=8; KO, n=9) and are representative of two independent experiments.  

C) Summary of EAE clinical data.  

<table>
<thead>
<tr>
<th>genotype</th>
<th>EAE incidence</th>
<th>Cumulative EAE Score</th>
<th>Maximum EAE score</th>
<th>Day of EAE onset</th>
<th>#Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ship^{+/+}</td>
<td>16/16</td>
<td>26.4 ± 23</td>
<td>3.5 ± 2.2</td>
<td>12.6 ± 3</td>
<td>2/16</td>
</tr>
<tr>
<td>Ship^{-/-}</td>
<td>15/17</td>
<td>27.4 ± 29</td>
<td>2.9 ± 2</td>
<td>15.7 ± 5.7</td>
<td>6/17</td>
</tr>
</tbody>
</table>
5.2.3 MOG\textsubscript{35-55}-induced responses are reduced in SHIP-deficient mice

To assess MOG-specific Ag recognition by T cells after EAE induction, we compared MOG-specific T cell proliferative responses and cytokine production of lymph node cells and splenocytes harvested 10 days after immunization. We found that proliferation of both LN cells and splenocytes re-stimulated with MOG\textsubscript{35-55} were greatly reduced in SHIP-deficient mice (Fig 5.3A and B, respectively). On the other hand, proliferation in response to polyclonal (αCD3 and αCD28) stimulation was similar between Ship\textsubscript{-/-} and WT cells (Fig 5.3C). As well, the production of IFN\textgamma in response to MOG stimulation was dramatically reduced in Ship\textsubscript{-/-} splenocytes compared to WT cells (Fig 5.3D), as was the production of IL-4 (Fig 5.3E). Since the production of IL-17 by MOG-primed T\textsubscript{17} cells was recently found to be essential for EAE development, we also assessed the level of IL-17 and found that MOG-induced production was greatly reduced in splenocytes isolated from SHIP-deficient mice (Fig 5.3F). Interestingly, the basal production of TNF\textalpha was very high from Ship\textsubscript{-/-} splenocytes but did not increase with MOG stimulation, whereas WT splenocytes secreted lower basal levels but reached higher levels that those of Ship\textsubscript{-/-} splenocytes with MOG stimulation (Fig 5.3G). There was no difference in the production of IL-10 from MOG-stimulated Ship\textsubscript{-/-} and WT cells (Fig 5.3H). Next, since NO production by inducible nitric oxide synthase (iNOS) has been shown to protect against EAE, ie., iNOS activity has been shown to increase during the course of EAE, correlating with clinical course, and the NO produced mediates protection since iNOS-deficient mice have exacerbated symptoms (Fenyk-Melody et al., 1998), we also determined NO levels in splenocyte culture supernatants. We found that Ship\textsubscript{-/-} splenocytes could not induce NO, while WT splenocytes showed a dose dependent increase in NO in response to MOG\textsubscript{35-55} (Fig 5.3I). These results are reminiscent of the M2 M\textgreekphi phenotype (high arginase, low iNOS) of SHIP-deficient mice.
Figure 5.3 Impaired MOG35.55-induced proliferation and cytokine production by T cells from immunized SHIP-deficient mice. A) Lymph node cells or B) Splenocytes prepared from WT or SHIP-deficient mice (-/-) 10 days after immunization with MOG35.55 were assayed for cell proliferation in response to the indicated dose of MOG35.55. C) Lymph node and splenic T cell proliferation responses to polyclonal activation, using anti-CD3 + anti-CD28, from WT and SHIP-deficient mice. D-I) Cytokine production from splenocytes isolated from MOG35.55 immunized WT and SHIP-deficient mice after stimulation with the indicated dose of MOG35.55. Data shown are means ± SEM from triplicate determination of three individual mice and are representative of two independent experiments. * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. Ship/-/- DCs).
5.2.4 Altered spleen cell composition in both naïve and EAE-induced SHIP-deficient mice

We next examined the splenocytes of WT and SHIP-deficient mice and found that naïve SHIP-deficient mice had increased numbers of splenocytes, in keeping with previous reports (Fig 5.4A)(Helgason et al., 1998). In addition, naïve SHIP-deficient mice had a reduced percentage of B220⁺, CD4⁺ and CD8⁺ cells. However, because of the increased number of total splenocytes in SHIP-deficient mice, there were no statistically significant differences in total cell numbers. On the other hand, the percentage of CD11c⁺ cells was increased in SHIP-deficient mice and this increase was enhanced when total cell numbers were taken into account (Fig 5.4B). After EAE induction, both WT and SHIP-deficient mice significantly increased their total number of splenocytes (Fig 5.4A). Also, as would be expected with the immune activation caused by EAE induction, WT and SHIP-deficient mice both had significant increases in their total numbers of CD4⁺ and CD8⁺ cells. However, SHIP-deficient mice increased their percentage and total number of B220⁺ splenocytes, resulting in SHIP-deficient mice having higher absolute number of B220⁺ cells than WT mice (Fig 5.4C).
Figure 5.4 Spleen cell compositions of naïve and EAE-induced WT and SHIP-deficient mice. A) Total nucleated cell counts of naïve and EAE-induced WT and SHIP-deficient mice. B & C) Percentage positive (left panel) and total number (right panel) of B220⁺, CD4⁺, CD8α⁺, CD11c⁺ and NK1.1⁺ cells in B) naïve and C) 10 day EAE-induced WT and SHIP-deficient mice. Data shown are mean ± SEM from naïve, n=3 (WT and Ship⁻/⁻) and EAE-induced, n=5 (WT) and n=4 (Ship⁻/⁻). * p<0.05; ** p<0.01; ‡ p<0.001 (WT vs. Ship⁻/⁻).
5.2.5 Splenic CD11b\(^+\) cells are increased in naïve and EAE-induced SHIP-deficient mice

CD11b\(^+\) cells from EAE-induced mice have immune suppressive activity \textit{in vitro} (Zhu et al., 2007). Thus, we assessed the presence of these cells in both naïve and EAE-induced WT and SHIP-deficient spleens. In WT mice, 2.9 ± 0.6\% of splenocytes were CD11b\(^+\) compared to 11 ± 1.5\% in SHIP-deficient mice (Fig 5.5A). In WT mice, the contribution of CD11b\(^+\) cells increased to 6.9 ± 1.1\% ten days after MOG\(_{35-55}\) immunization compared to 22 ± 6.4\% in SHIP-deficient mice. Taking the total number of splenocytes into consideration, WT and SHIP-deficient mice had 5.6 ± 1.7 \times 10^6 and 38.5 ± 7.7 \times 10^6 CD11b\(^+\) splenocytes in naïve mice, respectively (WT, n=5; KO, n=4). EAE-induced WT and SHIP-deficient mice had 20.9 ± 2.9 \times 10^6 and 135.9 ± 65 \times 10^6 CD11b\(^+\) cells, respectively (n=3). Since a previous study found that the suppressive cells within the CD11b\(^+\) population were Ly6C\(^{\text{high}}\)Ly6G\(^{\text{−}}\), we examined the distribution of Ly6C and Ly6G positive cells within the CD11b\(^+\) population of EAE-induced WT and \textit{Ship}⁻/⁻ splenocytes (Zhu et al., 2007). We found that a greater proportion of the CD11b\(^+\) cells from MOG\(_{35-55}\) immunized mice were also Ly6C\(^{\text{high}}\)Ly6G\(^{\text{−}}\) in SHIP-deficient mice (Fig 5.5B). Thus, EAE-induced \textit{Ship}⁻/⁻ mouse possess 38.2 \times 10^6 splenocytes with an ‘EAE disease suppressive’ Ly6C\(^{\text{high}}\)Ly6G\(^{\text{−}}\) phenotype while EAE-induced WT mouse spleens have nearly ten-fold fewer (3.9 \times 10^6) Ly6C\(^{\text{high}}\)Ly6G\(^{\text{−}}\) cells. Thus, the higher number of Ly6C\(^{\text{high}}\)Ly6G\(^{\text{−}}\) spleen cells of the SHIP-deficient mice may act to suppress MOG-induced T cell proliferation and thus might be responsible for the delayed onset of EAE compared to WT mice.
Figure 5.5 Splenic CD11b⁺ cells are increased in naïve and EAE-induced SHIP-deficient mice. A) Splenocytes were isolated from naïve WT and SHIP-deficient mice (left panels) or from day 10 post MOG35-55 immunized mice (right panels) and subjected to flow cytometry to determine the percentage of CD11b⁺ splenocytes pre- and post-immunization. B) Splenic CD11b⁺ cells from day 10 MOG35-55 immunized WT and SHIP-deficient mice were gated and analyzed by flow cytometry for the expression of Ly6G and Ly6C.
5.2.6 *Ship−/-* splenic DCs produce less IL-12 and are less able to induce Ag-specific T cell proliferation *in vitro*

DCs are critically important in priming naïve T cells. DCs first capture Ag and migrate to draining lymph nodes, during which time they undergo maturation. Once at a lymph node, DCs present MHC-bound peptides to T cells in the presence of co-stimulation and cytokine production (in particular IL-12) to induce an Ag-specific immune response. Since a previous study demonstrated reduced maturation of *Ship−/-* splenic DCs in response to LPS, as well as reduced allogeneic T cell responses (Neill et al., 2007), we assessed the ability of *Ship−/-* splenic DCs to secrete cytokines in response to LPS and to induce Ag-specific T cell proliferation. We found that IL-12 production was impaired from *Ship−/-* DCs, while the production of IL-6 and TNFα is enhanced, as is the production of IL-10, an anti-inflammatory cytokine (Fig 5.6A). We next examined the ability of *Ship−/-* DCs to induce OVA-specific CD4⁺ T cell (OTII transgenic) proliferation. WT and *Ship−/-* DCs were simulated with LPS and pulsed with varying concentrations of OVA₃₂₃₋₃₃₉ peptide for three hours. OTII CD4⁺ T cells were added to these cultures and proliferation was measured after 72 hrs by the incorporation of ³H-thymidine. The proliferation of OTII T cells cultured with *Ship−/-* DCs was markedly impaired compared to WT DCs (Fig 5.6B). This suggests that priming of Ag-specific T cell by DCs is defective in *Ship−/-* mice and likely accounts, in part, for the reduction of MOG-induced proliferation in the peptide recall responses of SHIP-deficient splenocytes.
Figure 5.6 Splenic CD11c+ DCs from SHIP-deficient mice are impaired in priming OVA-specific CD4+ T cells. A) CD11c+ DCs were purified from the spleens of WT and SHIP-deficient mice and stimulated for 24 hrs with LPS. Supernatants were collected and analysed by cytometric bead array. B) CD4+ T cells were isolated from OTII transgenic mice and cultured with LPS-activated OVA-loaded splenic DCs and the indicated concentration of OVA323-339. T cell proliferation was determined by 3H-thymidine incorporation. Results displayed are the mean ± SEM of triplicate determinations. Results are representative of at least two independent experiments.
5.2.7 SHIP-deficient mice produce higher amounts of anti-MOG IgM

Thus far, our mechanistic evidence would predict SHIP-deficient mice should be protected from EAE—in contrast with our observed disease course. Therefore, in search of a mechanistic explanation, we examined the B cell responses induced in MOG-primed WT and SHIP-deficient mice. Following immunization with MOG35-55, SHIP-deficient mice produced increased amounts of circulating anti-MOG IgM antibodies compared to WT mice after 10 and 60 days (Fig 5.7A). However, when the levels of anti-MOG IgG antibodies were compared, no significant differences were detected (Fig 5.7B). This illustrates a preferential expansion of anti-MOG IgM producing cells in SHIP-deficient mice or enhanced secretion of MOG-specific IgM, which could account for the level of disease induction observed in SHIP-deficient mice.

Figure 5.7 Serum levels of anti-MOG antibodies in WT and SHIP-deficient mice. WT and SHIP-deficient mice were immunized with MOG35-55. Mice were sacrificed and sera collected on day 10 and day 60 post immunization. A) IgM and B) IgG levels were measured by ELISA. Data shown are the means of two separate measurements of each sample. Horizontal bars indicate mean values and each data point represents one mouse.
5.3 Discussion

In this study we demonstrate a role for SHIP in the development of MOG_{35-55} induced EAE. SHIP-deficient mice developed a clinical disease course comparable to that of WT mice (Fig 5.2). A robust EAE-induction and disease course occurred in Ship^{-/-} mice despite the absence of a T cell proliferative response to MOG_{35-55} peptide. This decreased proliferative response was associated with reduced T_{H1} maturation towards T_{H1}, T_{H2} and T_{H17} phenotypes as well as reduced pro-inflammatory cytokine and NO production in the culture supernatants of MOG_{35-55}-primed splenocytes (Fig 5.3). In addition, increased numbers of suppressive IMCs were present in the spleens of EAE-induced SHIP-deficient mice (Fig 5.5). SHIP-deficient mice did, however, produce an increase in anti-MOG IgM Ab levels (Fig 5.7) which could account for the level of disease observed and, physiologically, overrides the coexisting disease-suppressive environment in Ship^{-/-} mice.

Although EAE was initially classified as a T_{H1}-mediated disease characterized by IFN\_\gamma secretion by T cells (Olsson, 1995), further study has revealed a role for the newly discovered T_{H17} cell in disease pathogenesis (Chen et al., 2006b; Cua et al., 2003). However, other studies have also found an important contribution from B cells in the development and progression of EAE (Cross et al., 2001; Matsushita et al., 2008). Contradictory roles for B cells in EAE pathogenesis have been demonstrated from protective to pathological (Cross et al., 2001; Du and Sriram, 2002; Fillatreau et al., 2002; Wolf et al., 1996). Early studies published on the effects of B cell depletion showed resistance to MBP-induced EAE (Gausas et al., 1982; Myers et al., 1992; Willenborg and Prowse, 1983) that could be overcome with the administration of serum from MBP-primed animals at the time of immunization (Willenborg et al., 1986). Although (induced) auto-antibody production is not essential for EAE induction, enhanced demyelination and inflammation can be caused by MOG-specific auto-Abs (Linington et al., 1988; Lyons et al., 1999a). Interestingly, some studies have found that the role of B cells is dependent on the immunogen used. For example, B cells were required for development of EAE when whole MOG protein was injected but dispensable
when MOG\textsubscript{35-55} peptide was used as the inoculant (Lyons et al., 1999a). The endogenous MOG Ag is a particularly good target for auto-Abs because, unlike PLP or MBP, it is expressed at the cell surface (Gardinier et al., 1992).

Another study which showed results analogous to our own were conducted with Lyn-deficient mice (Du and Sriram, 2002). Lyn is a Src family tyrosine kinase (SFK) that has been shown to phosphorylate SHIP in several immune cell types—and this phosphorylation may be important for SHIP's membrane recruitment and activity (Chan et al., 1997; Hibbs et al., 1995; Nishizumi et al., 1995). Perhaps because Lyn is an upstream regulator of SHIP activity, several similarities in the phenotypes of SHIP and Lyn-deficient mice have been described (Baran et al., 2003; Harder et al., 2004; Maxwell et al., 2004). In an EAE model, Lyn-deficient mice exhibited reduced T cell proliferation and T\textsubscript{H}1 cytokine secretion following MOG\textsubscript{35-55} immunization but enhanced production of anti-MOG IgM. The authors of this study attributed the exacerbation of disease in Lyn\textsuperscript{-/-} mice to this enhance IgM production (Du and Sriram, 2002). The primary difference between the results obtained with Lyn-deficient mice and our study with SHIP-deficient mice is that we saw similar rather than enhanced clinical scores compared to WT mice. These differences may be because of increased numbers of suppressive cells in SHIP-deficient mice, in particular Tregs (Collazo et al., 2009; Kashiwada et al., 2006) and IMCs (Fig 5.5), as well as reduced DC-induced T cell priming (Fig 5.6).

The presence of high numbers of CD11b\textsuperscript{+}Ly6C\textsuperscript{+} (IMC) cells in the spleens of SHIP-deficient mice after EAE induction (Fig 5.5) presents some intriguing questions. Cells of a similar nature (CD11b\textsuperscript{+}Gr1\textsuperscript{+}), termed myeloid derived suppressor cells (MDSCs), repress tumour immunity and have been shown to function in a similar fashion to the IMCs described in EAE, suppressing T cell proliferation and cytokine production through an iNOS/Arg1 dependent pathway (Geissmann et al., 2003; Nagaraj and Gabrilovich, 2008; Zhu et al., 2007). However, depending on the context, CD11b\textsuperscript{+}Ly6C\textsuperscript{+} cells have also been shown to be recruited to sites of inflammation where they can differentiate into DCs (Geissmann et al., 2003). In particular, in \textit{Listeria monocytogenes} infection, CD11b\textsuperscript{+}Ly6C\textsuperscript{+} cells develop into highly inflammatory TNF\textalpha- and iNOS-
producing DCs (Tip DCs) critical for bacterial clearance (Serbina et al., 2003b; Serbina and Pamer, 2006). In a very recent study, circulating CD11b+Ly6C+ cells from EAE-induced mice functioned more as their name (inflammatory monocytes) would suggest rather than suppressive MDSCs as their \textit{in vitro} characterization would have suggested (King et al., 2009). In addition, these cells were found to home to sites of CNS inflammation, cross the blood-brain barrier and acquire characteristics of Tip DCs. It is likely that the microenvironment in which CD11b+Ly6C+ cells are found dramatically impacts their function. Therefore, the presence of high numbers of CD11b+Ly6C+ cells in SHIP-deficient spleens could provide protection or exacerbation. Within the spleen, it would be expected that they produce NO and prevent the expansion of MOG-specific T cells. However, we did not detect high levels of NO from splenocyte cultures, suggesting that perhaps \textit{Ship-/-} IMCs, like MDSCs, express arginase 1 preventing NO production, but rather suppressing T cells by L-arginine depletion. Upon migration to the CNS, they adopt a highly inflammatory phenotype, increasing disease. However, it is also possible that the CNS environment is in constant flux, changing the function of CD11b+Ly6C+ cells from inflammatory during disease progression to suppressive during remission. In addition, it is also possible that the M2 (macrophage 'healer') environment present in naïve SHIP-deficient mice would create an environment that may maintain CD11b+Ly6C+ cells in a persistently suppressive state.

In conclusion, our results suggest that SHIP plays an important role in the development of EAE. Although typically classed as a T cell-mediated disease, we have found that, in the absence of SHIP, B cells play a prominent role in EAE progression, ie., production of auto-Abs in SHIP-deficient mice was sufficient to offset a deficient TH response to initiate disease. In addition, we found increased CD11b+Ly6C+ cells in the spleens of SHIP-deficient mice. These cells potentially inhibit autoreactive T cells or, alternatively, generate enhanced CNS inflammation. Further study will be required to fully dissect the relative importance of a variety of different cell types in the progression of EAE in SHIP-deficient mice. This will be made possible in future studies using tissue-specific SHIP deletion models. In particular, the comparison of EAE progression in a B cell specific SHIP deletion versus a macrophage/myeloid SHIP deletion will yield insight.
into the role of SHIP-deficiency in B cells compared to environmental factors that affect B cell phenotype (Karlsson et al., 2003; Nakamura et al., 2004).
CHAPTER 6: SHIP-DEFICIENT DENDRITIC CELLS SUPPRESS T CELL PROLIFERATION VIA A CONTACT-DEPENDENT, NITRIC OXIDE-INDEPENDENT MECHANISM

6.1 Introduction

The roles DCs play in the generation of immune responses have long been recognized. However, in addition to their importance in activating the immune system, DCs are also critical players in the induction of central tolerance (Zal et al., 1994) as well as the induction and maintenance of peripheral tolerance (Steinman et al., 2003). Several signals are involved in determining the nature of the interaction between T cells and DCs, including; the Ag-specific interaction between the TCR and the peptide-bound MHC molecule; contact mediated signals transduced by co-stimulatory molecules or tolerogenic molecules; and, secreted cytokines (Lange et al., 2007). Under normal steady state conditions, DCs maintain tolerance by either causing deletion or anergy of self-reactive T cells or inducing Tregs (Hawiger et al., 2001). Similar DCs can be generated in vitro, which have potential applications in animal models. A greater understanding of the mechanisms involved will allow tailoring of DCs for specific applications.

Immune suppression is not a function associated only with DCs. Several cell types including Tregs (Fontenot et al., 2003) and tumour-induced myeloid-derived suppressor cells (MDSCs), which are characterized by the co-expression of Gr1 and CD11b (Serafini et al., 2006), are capable of immune suppression. The mechanisms of suppression used by these cells can be quite diverse. Suppression by Tregs is often associated with either membrane-bound- or secreted TGFβ-induced anergy (Savage et al., 2008), cytokine deprivation-mediated apoptosis (Pandiyan et al., 2007) and/or contact-dependent cell death, involving granzyme B (Gondek et al., 2005). MDSCs, on the other hand, often use a different arsenal of suppressive mechanisms. In general, metabolism of L-arginine appears to play a central role. Arginine is metabolised by inducible nitric oxide synthase (iNOS, also known as NOS2), generating citrulline and NO, or it can be converted into urea and L-ornithine by the enzyme arginase 1 (Arg 1) (Bronte and
Zanovello, 2005a). Expression of these enzymes can lead to superoxide production, resulting in the generation of reactive nitrogen-oxide species (RNOS) such as peroxynitrite which, in turn, nitrosylates the TCR and other proteins, causing T cell suppression (Bronte et al., 2003).

DCs derived from rat BM using GM-CSF+IL-4 have been shown to have an intrinsic ability to prevent T cell proliferation while those derived using Flt3L do not (Taieb et al., 2007). However, the specific mechanism of GM-CSF+IL-4-derived DC-mediated suppression has not been determined. Another study, using myeloid dendritic cell (mDC) precursors, isolated as CD11c− cells generated in GM-CSF DC cultures, were found to have T cell suppressive properties. In this case, the mechanism of suppression was determined to be contact and NO dependent (Rossner et al., 2005). In addition, DCs that have been exposed to tumour cells become immunosuppressive via down-regulation of the TCR component CD3ε, and reactive oxygen species (ROS)-mediated apoptosis (Kuang et al., 2008). Taken together, these data illustrate a diversity of mechanisms available to DCs to induce T cell suppression. Importantly, the environment in which the DCs are generated appears to influence their ability to suppress as well as the suppressive mechanism they employ. SHIP is a critical negative regulator of the PI3K pathway with known functions in regulating myeloid cell development and survival. However, it is not known if it plays a role in the suppressive ability of DC subsets. In this study we co-cultured WT and SHIP-deficient DCs derived with GM-CSF (GM-DC), GM-CSF + IL-4 (GM/IL-4-DC) or Flt3L (FL-DC) as well as splenic isolated DCs with polyclonally activated T cells to determine the role of SHIP in DC-induced T cell suppression.

6.2 Results

6.2.1 GM-CSF-derived Ship−/- DCs express Arginase 1

L-arginine metabolism and Arg 1 expression has been shown to be an important part of T cell suppression mechanisms (Bronte and Zanovello, 2005; Rodriguez and Ochoa, 2008; Serafini et al., 2006). As well, Ship−/- peritoneal macrophages have been
shown to have an alternatively activated phenotype, characterized by Arg 1 expression, indicative of an immunosuppressive phenotype, and this has been linked to enhanced tumour growth in SHIP-deficient mice, (Rauh et al., 2005). Therefore, we examined the expression of Arg 1 in WT and Ship-/- DCs derived under different culture conditions (Fig 6.1A). We found that WT and Ship-/- DCs derived in the presence of GM-CSF and IL-4 express Arg 1. This finding is consistent with the literature which shows that IL-4 alone induces Arg 1 expression in BM-derived DCs (Corraliza et al., 1995; Louis et al., 1999; Modolell et al., 1995; Munder et al., 1998). Interestingly, when cultured with GM-CSF in the absence of IL-4, only Ship-/- DCs expressed Arg 1. We confirmed that increased arginase activity correlated with the level of Arg 1 detected by immunoblotting (Fig 6.1B). Meanwhile, neither WT nor Ship-/- DCs expressed Arg 1 when cultured with Flt3L (Fig 6.1A). This suggested that Ship-/- GM-DCs might be more suppressive than their WT counterparts.

**Figure 6.1 GM-CSF-derived Ship-/- DCs express Arg1.** A) Day 8 GM-CSF- (GM), GM-CSF+IL-4- (GM/IL-4) and Flt3L-derived DCs from WT (+/+) and Ship-/- (-/-) were subjected to Western analysis using Abs to SHIP, Arg1 and GAPDH. B) Arginase activity was determined in day 8 WT and Ship-/- GM-CSF ± IL-4-derived DCs, by measuring the amount of urea produced from the hydrolysis of L-arginine in 1 hr and then normalized to total protein (BCA assay). Data shown are representative of at least 3 independent experiments.
6.2.2 Both WT and Ship-/- GM-CSF ± IL-4-derived DCs suppress polyclonal T cell activation

To test whether WT or Ship-/- DCs isolated from the spleen or derived under different culture conditions had suppressive activity, we cultured different concentrations of these DCs with splenic T cells activated with αCD3 and αCD28. As shown in Fig 6.2A and 6.2B, WT and Ship-/- DCs isolated from the spleen or derived using Flt3L did not suppress T cell proliferation at any cell concentration tested. In contrast, WT and Ship-/- GM-CSF ± IL-4-derived DCs suppressed T cell proliferation in a cell dose dependent manner, with greater than 50% suppression achieved with the addition of 12.5x10³ DCs (Fig 6.2C and 6.2D). While only Ship-/- GM-DCs expressed Arg 1, there was no significant difference in the ability of WT and Ship-/- GM-DCs to suppress, nor did the addition of IL-4 to either culture significantly increase their suppressive ability.

Figure 6.2 Both WT and Ship-/- GM-CSF ± IL-4 derived DCs suppress polyclonal T cell activation while WT and Ship-/- splenic or Flt3L-derived DCs do not. 2x10⁵ WT splenocytes were stimulated with soluble αCD3 and αCD28 antibodies and incubated with the indicated number of either A) CD11c⁺ splenic DCs B) FL- C) GM- or D) GM/IL-4 DCs. Proliferation was determined after 72 hrs by incorporation of ³H-thymidine during last 18 hrs. Data shown are the mean ± SEM of triplicate cultures and are representative of more than 3 independent experiments.
In addition to T cell proliferation, we also analyzed cytokine secretion from αCD3 and αCD28 stimulated WT spleen cells co-cultured with WT or Ship-/- DCs isolated from the spleen or derived from BM in the presence of Flt3L or GM-CSF. We found that IFNγ, IL-10 and IL-17 production correlated with our T cell proliferation results, with no reduction being observed in the presence of WT or Ship-/- splenic DCs or FL-DCs (Fig 6.3A and 6.3B) and significant suppression of cytokine production achieved with WT and Ship-/- GM-DCs (Fig 6.3C). In addition to cytokines, we also determined the NO levels produced in the cultures and found that very little NO was produced in co-cultures of splenic or FL-DCs. However, co-cultures with WT GM-DCs showed a cell concentration dependent increase in NO production. Interestingly, Ship-/- GM-DCs secreted very little NO when large numbers of Ship-/- DCs were added (Fig 6.3C, bottom right panel). This result is in concordance with the Arg 1 expression in Ship-/- DCs, which prevents robust NO production by diverting the substrate, L-arginine, away from NO production, and also presents the possibility that WT and Ship-/- GM-DCs may suppress T cell proliferation/activation via different mechanisms.
Figure 6.3 WT and Ship-/− GM-CSF-derived DCs suppress cytokine production when co-cultured with activated T cells while WT and Ship-/− splenic or Flt3L derived DCs do not. WT splenocytes were stimulated with soluble αCD3 and αCD28 antibodies and incubated with the indicated number of either A) CD11c+ splenic B) FL- or C) GM-DCs. Supernatants were collected after 72 hrs and subjected to cytokine ELISA or Griess assay for NO determination. Data shown are the mean ± SEM of triplicate cultures and are representative of 2 or more independent experiments.
6.2.3 Suppression is not mediated by amino acid depletion or secreted factors

As shown in Fig 6.1A, we found that Ship-/- but not WT GM-DCs expressed the enzyme Arg 1. Others have found suppression of T cell proliferation can be achieved by other cell types through the expression of Arg 1 and subsequent depletion of the availability of L-arginine (Zea et al., 2005). Therefore, the possibility that Ship-/- GM-DCs were suppressing T cell proliferation through an arginase-dependent mechanism was explored. To test this, we used the Arg 1 inhibitor, BEC ([S]-[2-boronoethyl]-L-cysteine-HCl), and found that there was no reversal of suppression in either the WT or Ship-/- co-cultures (Fig 6.4A). In addition, we also added exogenous L-arginine to cultures and found no reversal of suppression. The amino acid tryptophan has also been recognized as an important limiting nutrient in the regulation of T cell responses (Fallarino et al., 2002). Tryptophan is the rarest essential amino acid and may cause a ‘bottle-neck’ in protein synthesis. Local depletion of tryptophan can cause the death of T cells, whose survival and expansion are dependent on this amino acid (Mellor and Munn, 1999). In addition, tryptophan catabolites can lead to T cell apoptosis (Terness et al., 2002). The enzyme indoleamine 2,3-dioxygenase (IDO), is a key enzyme of tryptophan degradation and is expressed by some DC subsets (Fallarino et al., 2002). To test if IDO was the cause of the suppressed T cell proliferation we added an IDO inhibitor, exiguamine A (Carr et al., 2008), to cultures. We found that inhibiting IDO had no effect, nor did the addition of exogenous L-tryptophan (Fig 6.4A).

In addition to depletion of amino acids in the media, suppression is also often mediated by secretion of cytokines into the media. Therefore, we neutralized several cytokines with known or potential suppressive functions. Addition of neutralizing antibodies to IL-4, TGFβ, IL-13, IL-6 and IL-10 resulted in no significant differences compared to an isotype control antibody (Fig 6.4B). TGFβ is often membrane bound and expressed at the cell surface in a latent form due to its non-covalent association with latency associated peptide (LAP). Removal of LAP allows the release of bioactive TGFβ (Saharinen et al., 1999). Addition of commercial exogenous LAP to retain TGFβ in an inactive form had no effect on the level of suppression (Fig 6.4C). These results suggest
that DC secreted cytokines are not responsible for the suppression of either WT or Ship/- GM-DCs.

Figure 6.4 Neither amino acid depletion nor secreted cytokines are responsible for T cell suppression. WT splenocytes were stimulated with soluble αCD3 and αCD28 Abs and incubated with WT or Ship/- GM-DCs (50x10^3) with or without: A) arginase inhibitor (BEC, 100 μM), L-arginine supplementation (L-Arg, 2 mM), IDO inhibitor exigamine A (Exi, 1 μM) or L-tryptophan supplementation (L-tryp, 200 μM) B) isotype control Ab (iso) or the indicated neutralizing cytokine Ab (10 μg/ml). Values represent the mean ± SEM of the relative percent of suppression (calculated by the ratio of suppression with the indicated treatment relative to the suppression in the absence of treatment—see Materials and Methods for formula) from triplicate determinations and is representative of at least two independent experiments. C) The percent suppression of WT splenocytes stimulated with αCD3 and αCD28 Abs in the presence of LAP (250 ng/ml). Data shown are the mean ± SEM of triplicate determinations and are representative of 2 independent experiments.
6.2.4 WT and *Ship*-/ GM-CSF-derived DC suppression is not mediated by an IL-2-dependent mechanism

IL-2 is an important cytokine for T cell proliferation. We therefore determined if the suppression of T cell proliferation induced by WT and *Ship*-/ GM-DCs was occurring via inhibition of autocrine-acting IL-2 production. Surprisingly, we found the opposite. In fact, we observed the greatest suppression in cultures with the highest levels of IL-2 (Fig 6.5A, left panel). Since this result was quite unexpected, we also confirmed it using qRT-PCR, in order to rule out the possibility that in the cultures where the T cells were proliferating rapidly they were simply binding up and internalizing all of the IL-2 generated, rendering it unavailable for detection by ELISA. This, however, proved not to be the case since IL-2 mRNA levels were also highest in the most suppressed samples (Fig 6.5A, right panel). In light of the enhanced IL-2 production in suppressed samples, the next possibility we investigated was that WT and *Ship*-/ GM-DCs were inhibiting the expression of CD25 (the IL-2R α-chain) and thus blunting responsiveness to IL-2. Using flow cytometry, we measured CD25 expression levels on CD4+ cells within the co-cultures. We found that, much like the IL-2 levels, expression of CD25 was also greatest in the most suppressed samples, and showed a dose dependent increase with number of DCs added (Fig 6.5B). Lastly, to rule out an IL-2 dependent mechanism of T cell suppression, we added exogenous IL-2 to the co-cultures of WT and *Ship*-/ GM-DCs and found that addition of IL-2 did enhance T cell proliferation in the absence of DCs, but did not abrogate suppression when DCs were present in the culture (Fig 6.5C). Taken together, these results rule out IL-2 availability being responsible for WT and *Ship*-/ GM-DC-induced T cell suppression.
Figure 6.5 Suppression by WT and Ship-/- GM-CSF-derived DCs is not alleviated by the addition of IL-2. WT splenocytes (Splen ctrl) were stimulated with soluble αCD3 and αCD28 Abs and incubated with the indicated number of WT (+/+ ) and Ship-/- GM-DCs and A) IL-2 levels in the supernatant determined after 72 hrs (left panel) or IL-2 mRNA levels determined by qPCR (right panel). B) CD4+ T cells were analysed by flow cytometry for expression of CD25. C) IL-2 (100 U) was added (or not) as indicated and proliferation determined after 72 hrs. Each point is the mean ± SEM of triplicate cultures and is representative of at least 2 independent experiments.
6.2.5 WT and *Ship/-/-* GM-CSF-derived DCs suppress via a contact-dependent mechanism

T cell suppression can be mediated by soluble cytokines, by direct contact, or both. To determine if suppression was contact dependent, since it did not appear to be mediated by secreted cytokines, we performed a transwell experiment. As can be seen in Fig 6.6A, separation of activated T cells from WT and *Ship/-/-* DCs by a semi-permeable transwell membrane abrogated suppression at all cell concentrations tested. To determine if certain contact molecules were responsible for the suppression, we blocked either the inhibitory receptor CTLA-4 or the adhesion molecules, LFA-1 and Mac-1 (CD11b), with neutralizing Abs. Under both conditions no change in the level of suppression was detected (Fig 6.6B). ROS have been implicated in phagocyte-induced T cell suppression (Kusmartsev et al., 2004), and although not necessarily requiring direct cell contact, close proximity is required due to the short half-life of these molecules. To determine if ROS was involved in either WT or *Ship/-/-* GM-DC-induced suppression, we added the ROS scavengers N-acetyl-cysteine (NAC), catalase and superoxide dismutase (SOD) to DC and activated T cell co-cultures. As shown in Fig 6.6C, addition of these ROS scavengers had no effect on T cell proliferation. These results demonstrate that although the mechanism of suppression is contact dependent, it is not ROS dependent.
Figure 6.6 WT and Ship−/− GM-CSF-derived DCs suppress via a contact-dependent mechanism. A) The indicated number of WT and Ship−/− GM-DCs were plated in the bottom chamber of a 0.4 μm 96 well transwell plate and WT splenocytes (2x10^5 c) were stimulated with soluble αCD3 and αCD28 Abs and plated in the top chamber. Proliferation was determined after 72 hrs by incorporation of ^3^H-thymidine for the final 18 hrs. Data shown are the mean ± SEM of triplicate cultures and are representative of 3 independent experiments. B) Relative percent suppression with the addition of blocking antibodies to CTLA4 (10 μg/ml) and LFA1+mac1 (5 μg/ml) C) Relative percent suppression with the addition of agents that reduce the presence of ROS (NAC, 2 mM; catalase, 100 U/ml; SOD, 200 U/ml). Data shown are the mean ± SEM of triplicate cultures and are representative of at least 2 independent experiments.

6.2.6 WT, but not Ship−/−, suppression is mediated by an IFNγ-regulated mechanism involving the production of NO

Other contact-dependent mechanisms that have been shown to be employed to suppress T cell proliferation, at least by MDSCs, have involved pathways downstream of IFNγ production (Movahedi et al., 2008; Rossner et al., 2005). When we added a neutralizing Ab to IFNγ, we found that WT GM-DCs were significantly less capable of
suppressing T cell proliferation and that less NO was generated in culture supernatants (Fig 6.7A and 6.7B). No effect was observed in Ship-/- cultures which fits with the inability of Ship-/- DCs to induce NO when added at 50 x 10³ cells, likely as a result of high Arg 1 activity. To determine if the reduced NO was involved in the contact-dependent suppression of WT GM-DCs, we used both an NO scavenger, carboxy PTIO, and an iNOS inhibitor, L-NMMA. As can be seen in Fig 6.8A, addition of carboxy PTIO significantly reduced the level of suppression, as did the addition of L-NMMA in a dose dependent manner for WT GM-DCs. The combination of the two compounds caused the most dramatic reduction in the level of suppression. The reduction in suppression induced by carboxy PTIO also correlated with a reduction in NO production (Fig 6.8B) as well as a complete prevention of NO produced with the addition of L-NMMA at all doses tested (data not shown). As expected, the addition of PTIO, L-NMMA or a combination of the two had no effect on the suppression induced by Ship-/- GM-DCs. These data indicate that WT GM-DCs suppress T cell proliferation via a contact-
dependent, IFNγ-induced production of NO. In the case of Ship-/- GM-DCs the mechanism is also contact-dependent, but does not involve IFNγ or the production of NO.

Figure 6.8 NO production is responsible for mediating WT GM-CSF-derived DC induced T cell suppression but Ship-/- DCs use an NO-independent mechanism. WT splenocytes (Splen control) were stimulated with soluble αCD3 and αCD28 Abs and incubated with WT (+/+) or Ship-/- GM-DCs (50x10^3) for 72 hrs. 

A) Relative percent suppression of proliferation with the indicated treatment (PTIO (25 μg/ml) + L-NMMA (0.5 mM)).

B) NO production with the addition of carboxy PTIO (25 μg/ml). Data shown are the mean ± SEM of triplicate cultures and are representative of 3 independent experiments. *p<0.05; **p<0.01; ‡p<0.001 relative to control, ns = not significantly different.
6.3 Discussion

In this study we have compared the ability of splenic as well as GM-CSF ± IL-4 and Flt3L-derived WT and *Ship*-/- DCs to suppress polyclonal T cell proliferation. We have demonstrated that both WT and *Ship*-/- DCs derived with GM-CSF ± IL-4 have an intrinsic ability to suppress T cell proliferation while Flt3L-derived and splenic DCs do not. Upon further investigation of the mechanism of suppression employed by GM-DCs, we discovered that WT cells utilized contact-dependent, IFNγ-induced NO production to suppress (see Model, Fig 6.9). On the other hand, *Ship*-/- GM-DCs did not produce significant amounts of NO at high DC concentrations, and suppression could not be reversed through neutralization of IFNγ, inhibition of iNOS or arginase. Suppression was, however, prevented when contact was abolished.

A number of studies have been conducted to investigate the influence of DC culture systems on their ability to suppress T cell activation *in vitro*. Based on these studies, the dose of GM-CSF used in culture as well as the addition of IL-4 was found to impact the resultant phenotype (Lutz et al., 2000b). Specifically, BMDCs derived under conditions where low doses of GM-CSF were employed were phenotypically less mature and could induce T cell unresponsiveness. In addition, these cells were much more resistant to LPS, TNFα and CD40-induced maturation, but were sensitive to the effect of IL-4-induced maturation. On the other hand, DCs derived with high doses of GM-CSF were more mature and showed little phenotype/functional difference with the addition of IL-4 (Lutz et al., 2000b). These results could be used to explain our finding that the addition of IL-4 to our cultures had no effect on suppressive ability, neither increasing nor decreasing it (Fig 6.2C and D). In our studies, DCs were cultured in the presence of high doses (10 ng/ml) of GM-CSF, resulting in the DCs being refractory to IL-4-induced effects. However, IL-4 does have a dual role in that it can induce both maturation (Lutz et al., 2000b) and the expression of Arg 1 (Corraliza et al., 1995; Louis et al., 1999; Modolell et al., 1995; Munder et al., 1998) which could potentially increase the suppressive activity of DCs. However, we did not find that either of these potential effects impacted the level of suppression found in our assay. The
Figure 6.9 Model of WT and Ship-/- GM-CSF-derived DC-induced T cell suppression. WT and Ship-/- GM-DCs both suppress T cell proliferation in a contact-dependent manner. WT GM-DCs induce NO secretion through an IFNγ-dependent mechanism, which results in T cell suppression. Ship-/- GM-DCs express Arg 1 and do not produce NO, but may use an alternate direct mechanism of suppression or induce the expansion or differentiation of a regulatory cell, possibly Tregs, to suppress T cell proliferation. If a second cell type is involved in suppression, its induction or activation is contact-dependent.
studies described (Lutz et al., 2000a; Lutz et al., 2000b) did not elucidate a mechanism of action of T cell unresponsiveness beyond the suggestion that the immature phenotype prevented activation. A subsequent study by the same investigators found that the non-DC fraction (CD11c⁻) of 8-10 day low GM-CSF cultures and 3-4 day high GM-CSF cultures suppressed T cell activation via a contact and NO-dependent mechanism (Rossner et al., 2005). This group classified these cells as in vitro-derived MDSCs (or MSCs, myeloid suppressor cells) whose in vivo suppressive function has been well characterized (Gabrilovich and Nagaraj, 2009). These results are strikingly similar to our results using the CD11c⁺ fraction from day 8 WT high concentration GM-CSF cultures. Although Lutz et al. did not investigate the suppressive potential of the CD11c⁺ cells in their cultures they did find that DCs generated from Gr1low cells were not suppressive. In addition, it has been reported that BM-derived rat DCs but not splenic isolated rat DCs produce NO and are capable of T cell suppression (Powell et al., 2003). Unfortunately, as already mentioned, several factors can influence the DCs generated, including GM-CSF concentration as well as factors such as age of mice used, starting cell density, mechanical stress and batch to batch variation in fetal calf serum (Lutz and Rossner, 2007). As well, the use of recombinant GM-CSF versus GM-CSF from conditioned media can render literature comparisons of results difficult. In addition, the mechanism of T cell activation also influences the relative ability of different cells to suppress as was seen in the case of different MDSC populations (Movahedi et al., 2008). However, a study using rat BM-derived DCs does confirm our result that WT GM/IL-4- but not FL-DCs suppress T cell responsiveness (Taieb et al., 2007), but a mechanism was not determined.

Intriguingly, our results demonstrate that DCs generated in the presence of GM-CSF from Ship⁻/⁻ BM are capable of suppression, but that this suppression is not reversible by any means tested, including those that reversed the suppressive activity of WT DCs. We did observe, however, that co-culture with either WT or Ship⁻/⁻ GM-DCs resulted in increased expression of CD25 on CD4⁺ cells (Fig 6.5B). It is therefore possible that contained within the CD25⁺ cell population are Tregs (co-expressing Foxp3) since DCs are known to expand CD4⁺CD25⁺Foxp3⁺ cells (Yamazaki et al., 2006). A
study using human DCs illustrated that regulatory DCs induced CD4^+CD25^+ Tregs which were capable of suppressing T cell responses (Sato et al., 2003a). This suggests that DC-induced suppression may not always be a direct effect, but rather mediated through the induction or expansion of Tregs (see Model, Fig 6.9). Based on our CD25 expression results as well as preliminary results investigating the presence of CD25^+Foxp3^+ Tregs in our co-cultures, we hypothesize that both WT and Ship^-/- GM-DCs induce Tregs, but Ship^-/- DCs are more reliant on them for their suppressive ability since they lack another mechanism of suppression. As well, the increase in IL-2 detected in suppressed cultures could point to Tregs because proliferation of Tregs has been shown to be partially dependent on IL-2 secretion by DCs and/or T cells (Yamazaki et al., 2003). Complicating matters, however, is the fact that we have already ruled out several Treg mechanisms of suppression, in particular secreted or membrane bound TGFβ (Fig 6.4B and C). In a recent study by Savage et al (Savage et al., 2008), alternatively activated Mφs induced Tregs which then suppressed T cell proliferation in a contact-dependent manner via membrane bound TGFβ. However, this study did not identify a mechanism of Treg induction. We can say that if Treg induction/expansion is responsible for the suppression by Ship^-/- GM-DCs that the mechanism of Treg induction is contact dependent and neither their induction nor their suppressive ability involves TGFβ. Further investigation will be required to confirm the role of Tregs and determine their mechanism of induction and action.

In conclusion, we show that, unlike Flt3L-derived and splenic isolated DCs, GM-CSF ± IL-4-derived WT and Ship^-/- DCs are capable of suppressing polyclonal T cell proliferation. It appears that WT GM-DCs suppress via a contact and IFNγ-dependent induction of NO while Ship^-/- DCs are incapable of NO production due to high expression of the enzyme Arg 1, yet are still equally suppressive. We believe this is the first example showing Arg 1 expression in DCs in the absence of exogenous IL-4. Thus far, the absence of a reversible mechanism of suppression for Ship^-/- GM-DCs suggests that they could be particularly good at preventing graft versus host disease or prolonging allograft survival in mice due to the reduced likelihood that they will be converted to immunogenic DCs in vivo. This finding could be applicable to a clinical setting through
the use of either inhibitors of SHIP or the use of RNA interference to reduce SHIP levels in human BM-derived DCs prior to transplant. Further understanding of the unique mechanism of T cell suppression by *Ship*−/− DCs will likely reveal other targets for the pharmacological manipulation of DC suppressive functions.
CHAPTER 7: SUMMARY AND PERSPECTIVES

The overall objective of the work presented in this thesis was to learn more about DC biology with a specific focus on the role of SHIP in innate activation, disease processes and T cell suppression. In Chapters 3 and 4 we explored the role of SHIP in the development and function of DCs derived from mouse BM in vitro with GM-CSF or Flt3L. We found a common function for SHIP in the differentiation of GM- and FL-DCs but unique roles for SHIP in the regulation of TLR-induced activation of these DC cultures. GM-DCs have long been used as the standard model for in vitro studies of DC biology, yet recent studies suggest that FL-DCs are likely more representative of steady state DCs whereas GM-DCs more closely recapitulate an inflammatory state DC. Therefore, our finding that the function of SHIP differs in GM- and FL-DCs in response to TLR activation suggests that 1) it is difficult to translate in vitro results to in vivo responsiveness 2) the function of SHIP in DCs may be different in steady state and inflammatory DCs and 3) one should consider the purpose of the study when selecting a source of DCs for investigation.

Consistent with previous studies (Xu et al., 2007), we found that WT GM-DCs were capable of producing TNFα (Fig 3.6) in response to LPS stimulation while only SHIP-deficient FL-DCs could produce significant amounts of TNFα (Fig 4.5). Thus, SHIP restrains LPS-induced TNFα production as well as the production of other cytokines in an in vitro model of ‘steady-state’ DCs. On the other hand, the production of IL-12 in response to LPS was enhanced by SHIP in GM-DCs, implicating SHIP as a positive regulator of inflammation in DCs specialized for pathogen clearance. These functional differences, to some extent, make intuitive sense. When in a resting steady state it is important to have mechanisms in place that limit unnecessary immune responses but, when an infection has occurred, specialized DCs are induced for the purpose of generating an inflammatory response and it is important that they be able to carry out this duty.
The primary function of DCs is to activate T cells and direct adaptive immune responses. We found that, despite differences in cytokine production by Ship-/- GM- and FL-DCs, both Ship-/- DCs were impaired in their ability to activate Ag-specific T cell proliferation—this functional defect correlated with defective TLR-induced maturation (based on phenotypic surface markers). Most notably, the functions and phenotype of Ship-/- FL-DCs were only significantly different from those of WT FL-DCs when stimulated with TLR agonists that acted through MyD88-independent pathways. For example, in FL-DCs, SHIP negatively regulated type I IFN and cytokine production but positively regulated maturation in response to activation of MyD88-independent signalling pathways. This finding contrasts with analogous work carried out with macrophages where SHIP induction was shown to be limited to MyD88-dependent pathways (Sly et al., 2009). Although we have not identified a mechanism by which SHIP affects MyD88-independent signalling, the prospect that SHIP serves a unique function in regulating the maturation of DCs and, ultimately, T cell proliferation, only in this arm of TLR-induced activation has important implications for the understanding of DC biology and the prospect of manipulating DC functions for clinical benefit. Unfortunately, very little is known about TLR-induced signalling in FL-DCs. In general, unique negative regulators of the MyD88-independent pathway that have been identified to date include cis-trans peptidylprolyl isomerase, NIMA-interacting 1 (PIN1) which inhibits IRF3 activation, SHP2, which inhibits TRIF-dependent cytokine and interferon induction, and SARM, which is a specific negative regulator of TRIF (Kawai and Akira, 2007). Complicating matters, however, is the fact that SHIP both negatively and positively regulates distinct aspects of MyD88-independent signalling. This dual role suggests that increases in PIP3 levels or decreases in PI-3,4-P2 levels, which occur in the absence of SHIP, modulate the activity of proteins that regulate MyD88-independent pathway components. Thus, changes in these phosphatidylinositides may lead to changes in cell surface expression of co-stimulatory molecules, IRF3 activation and pathways that lead to cytokine production. Clearly, further study is needed to dissect out the precise mechanism(s) by which SHIP exerts its effects in DCs.
We have also shown, for the first time, that SHIP-deficient mice fail to establish a Th1 response and have a Th2 bias (Chapter 3). Of note, these *in vivo* results correspond best with those obtained *in vitro* with GM-DCs (inflammatory model) and not FL-DCs (steady state model). *In vitro*, *Ship*-/- GM-DCs failed to skew T cells to a Th1 response while *Ship*-/- FL-DCs enhanced Th1 responses. This may be because immunization in the context of CFA (adjuvant) induces inflammatory DCs at the site of injection which then migrate to the spleen and are responsible for the priming of naïve T cells *in vivo*—thus resulting in similarities to *in vitro* results obtained using GM-DCs. This result suggests that specific pharmacological manipulation of SHIP activity *in vivo* may permit control of the response to immunization.

Although many of our results are intriguing, delineating the mechanisms underlying SHIP’s effects are complicated by the pan-hematopoietic and developmental impact that SHIP deletion has in our models. This is because the environment in which cells develop plays a large part in shaping their ultimate phenotype and functions. Since both our *in vivo* and *in vitro* DC studies were conducted in the presence of SHIP-deficient non-DCs (presumably also having altered phenotypes) it is possible they may influence DC development and function. For example, it is possible that the SHIP-deficient environment caused the more immature DC phenotype we observed and that, if this exogenous influence was eliminated, different results might be obtained. In addition, as described in a study using a T cell-specific SHIP deletion model (Tarasenko et al., 2007) it is possible that part of the reason SHIP-deficient mice failed to establish a Th1 response was a failing of the T cells themselves. Thus, in an *in vivo* environment with only SHIP-deficient DCs, we may find that the results are more similar to those obtained with FL-DCs ie., SHIP-deficient DCs actually skew T cells to a Th1 response. Thus, for the reasons cited above, it will be important in future studies to determine which of our findings are DC-autonomous by using DC-specific SHIP-deletion models. Such a model has recently been made available with the generation of a transgenic mouse line expressing the Cre recombinase under the control of the CD11c promoter (integrin α x (Itgax)) (Caton et al., 2007). When appropriately bred to a transgenic line with loxp-flanked *Ship* alleles (Paraiso et al., 2007), the resulting model (*Cd11cCre.Ship^loxP/loxP^*)
will allow specific deletion of SHIP in cells expressing CD11c at some point in their development (ie, primarily the DC lineage).

Based on the immaturity and resultant T cell stimulatory defects observed with \textit{in vitro} derived \textit{Ship}\textsuperscript{-/-} DCs, the finding that SHIP-deficient mice fail to skew to a T\textsubscript{H1} response (and the knowledge that macrophages in \textit{Ship}\textsuperscript{-/-} mice are M2 skewed), we wanted to determine how these factors affect the onset and course of experimental autoimmune encephalomyelitis (EAE) (Chapter 5). This mouse model of multiple sclerosis has been reported to be regulated by DC priming of naïve T cells. One study using Src homology 2 domain containing protein tyrosine phosphatase substrate-1 (SHPS-1) mutant mice, which is selectively expressed in DCs, found that these mice were resistant to EAE and that this was largely due to a defect in the ability of DCs to prime T cells (Tomizawa et al., 2007). Therefore, we hypothesized that we would observe a similar protective effect in SHIP-deficient mice. As described in Chapter 5, however, we found that SHIP-deficient mice developed EAE, despite our finding that splenic DCs, much like \textit{in vitro} derived DCs, have a reduced ability to prime Ag-specific T cell responses. As well, T cells isolated from EAE-induced \textit{Ship}\textsuperscript{-/-} mice exhibited reduced peptide recall ability and failed to become activated T\textsubscript{H} cells. Further investigation revealed that the EAE disease in SHIP-deficient mice was likely the result of enhanced B cell activation and overproduction of anti-MOG IgM\textsubscript{s}. As discussed above, further studies of the EAE disease course in SHIP-deficient mice will benefit from the use of cell type-specific SHIP deletion models. For example, a study by another group has shown that part of the B cell phenotype in ubiquitous \textit{Ship}\textsuperscript{-/-} mice is dependent on macrophages. One of the highlights of this study illustrated, using a myeloid/macrophage specific \textit{Ship}-deletion model (LysMCre.\textit{Ship}\textsuperscript{loxP/loxP}), that these mice maintain the defective marginal zone B cell phenotype of ubiquitous \textit{Ship}\textsuperscript{-/-} mice (Karlsson et al., 2003). Therefore, a comparison of a B cell and macrophage specific \textit{Ship}-deletion mouse models in an EAE disease course would determine if the disease phenotype is due to macrophage effects on B cells or B cell autonomous defects, resulting in hyper-production of anti-MOG IgM.
In our final study (Chapter 6), we investigated the suppressive abilities of different WT and *Ship*-/- DC subsets. We found that GM ± IL-4-DCs were capable of suppressing T cell proliferation but Flt3L-derived and splenic DCs were not. The suppressive ability of these GM-CSF ± IL-4 derived DCs did not correlate with Arg 1 expression or activity since both WT and *Ship*-/- GM-DCs were equally suppressive yet only *Ship*-/- DCs expressed Arg 1. In addition, inhibition of Arg 1 activity did not affect suppression. In an attempt to define the mechanisms of suppression, we tested several known mechanisms of T cell suppression, including secreted cytokines (TGFβ, IL-6, IL-4, IL-13 and IL-10), production of ROS and expression of inhibitory surface molecules. However, we found that blocking any of these mechanisms did not interfere with the suppressive function of either WT or *Ship*-/- GM-DCs. However, we did find that cell-cell contact was necessary for suppression. This result led us to the finding that αCD3 and αCD28-induced T cell release of IFNγ likely triggered NO production from WT DCs and this was necessary for WT-induced suppression. This NO-dependent suppressive mechanism concurs with reports in the literature using DC precursor cells and rat BM-derived DCs (Rossner et al., 2005; Taieb et al., 2007). Unexpectedly, however, *Ship*-/- GM-DCs did not use the same NO-dependent mechanism as WT GM-DCs. *Ship*-/- GM-DCs express high levels of Arg 1 and, therefore, do not produce appreciable levels of NO. Despite this finding, we discovered that suppression still required contact between *Ship*-/- DCs and activated T cells. Our current hypothesis is that *Ship*-/- GM-DCs induce Tregs through a contact-dependent but TGFβ-independent mechanism. In support of this, our preliminary results have shown that *Ship*-/- GM-DCs increase, in a cell concentration dependent manner, the proportion of CD4+Foxp3+ cells (Tregs). Further studies will be required to confirm this finding and conclusively demonstrate that *Ship*-/- GM-DC-induced Tregs are capable of suppressing T cell proliferation. In addition, since TGFβ has already been ruled out as a requirement for suppression, a different mechanism for Treg-mediated T effector cell suppression will need to be elucidated.

Our work exploring the mechanisms underlying DC function in innate and adaptive immunity has implications for treating human disease. For example, GM-DCs derived from a recipient could be used to either prevent rejection of donor solid organs,
or, GM-DCs derived from the donor could be used to prevent graft versus host disease by suppressing alloreactive T cells. Similar intervention strategies using DCs to prolong heart allograft survival in mice have had some success (Lutz et al., 2000b). With our contributions to the understanding of DC biology, particularly the role of SHIP in modulating DC maturation and function, further advances in DC-based therapies may benefit from targeting SHIP activity. Our results suggest that SHIP-deficient DCs have a suppressive phenotype, resistant to inhibition or reversion. Thus, functionally blocking SHIP activity may improve the long-term success of suppressive therapies.

The therapeutic use of DCs also has applications in cancer immunotherapy since DCs play a pivotal role in the initiation, programming and induction of anti-tumour immune responses (Melief, 2008). The immune system's role in cancer prevention is multifaceted. It has come to light in recent years that viruses are responsible for up to 15% of cancers in immunocompetent people and the ability to clear viral infections (e.g., human papilloma virus (HPV), a cause of cervical cancer) reduces the risk of developing certain cancers. DCs are critical activators of anti-viral immune responses and DC deficiencies result in reduced T cell responses and minimal viral clearance – the consequence may be initiation of virus-mediated cancers. Furthermore, 15-20% of malignancies are associated with chronic inflammation (e.g., inflammatory bowel disease contributing to colorectal cancer) (Mantovani and Pierotti, 2008). Proper control of inflammation reduces the likelihood of such cancers developing. Lastly, appropriate DC surveillance for transformed cells allows for detection and elimination of abnormal cells. Tumours, however, may acquire mechanisms to suppress DC function in the tumour environment (Gabrilovich, 2004). The therapeutic use of DCs in cancer has focused on DC cancer vaccines. Protocols have been developed to deliver tumour associated antigens (TAAs) Ags to human monocyte-derived DCs and various strategies have been evaluated in clinical trials (Dunn et al., 2004). Unfortunately, TAAs are not as robust as micro-organisms in triggering DC activation, likely because they are typically ‘self-like’ Ags. Thus TAA activation of DCs can lead to minimal maturation and the induction of tolerance rather than tumour-eliminating immune activities. Our finding that Ship-/- DCs have reduced maturation in response to innate signals and, consequently, reduced Ag-
specific T cell activation ability suggests that full elucidation of SHIP's role in DC function will contribute to new strategies using *ex vivo* generated DCs for immunotherapy.

In this thesis, we have elucidated the function of SHIP in the development and TLR-induced activation of DC subsets. We have also shown that, although DCs are powerful activators of immune responses, they can also suppress T cell proliferation and that SHIP is one of the key regulators of this suppressive function. Lastly, we found that SHIP-deficient mice develop EAE even in the presence of suppressive myeloid cells and reduced T cell activation, most likely via enhanced B cell production of IgM. Further work refining SHIP's role in specific cell lineages, and as part of an immune regulator network, will allow the development of cell-based therapies using *in vitro* derived DCs or treatments that manipulate SHIP/PI3K pathways in DCs *in vivo*.
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ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A05-0256

Investigator or Course Director: Gerald Krystal

Department: Pathology & Laboratory Medicine

Animals:

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<tr>
<td>C3H/HeN 200</td>
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<tr>
<td>C57B6 SHIP+/- mice 550</td>
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<td>Mice BalbC 200</td>
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<tr>
<td>Mice C57BL6 (SHIP+/+ 375, +/- 750, -/- 375, MyD88 +/- 100, +/- 100, 50 OT-11 1000</td>
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Approval Date: April 24, 2008

Funding Sources:

- **Funding Agency:** National Cancer Institute of Canada
  - **Funding Title:** The role of SHIP in hemopoietic cell proliferation, activation and transformation

- **Funding Agency:** National Cancer Institute of Canada
  - **Funding Title:** Regulation of proliferation versus differentiation during normal & leukemic hemopoiesis

Unfunded title: The Role of SHIP in hemopoiesis and innate immunity

The Animal Care Committee has examined and approved the use of animals for the above breeding program.
This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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ANIMAL CARE CERTIFICATE

Application Number: A07-0503

Investigator or Course Director: Gerald Krystal

Department: Pathology & Laboratory Medicine

Animals:

- Mice SHIP-/- C57BL6 25
- Mice OTII transgenic C57BL6 mice 30
- Mice C57BL6 15
- Mice SHIP+/+ C57BL6 25

Start Date: October 1, 2007

Approval Date: March 26, 2009

Funding Sources:

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- Funding Title: The role of SHIP in the generation and function of tumour associated macrophages.

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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